Department of Laboratory Medicine, Division of Clinical Microbiology

Karolinska Institute, Stockholm, Sweden

EFFECT OF VIRULENCE FACTORS ON SURVIVAL STRATEGIES OF *VIBRIO CHOLERAE* AND *VIBRIO MIMICUS*

Soni Priya Valeru

Stockholm 2013

Published by Karolinska Institutet. Printed by [Larserics Digital Print AB]

ISBN 978-91-7549-201-8

"Our greatest glory is not in never falling, but in rising every time we fall"

__Confucius

To the Lord God Almighty……

ABSTRACT

Vibrio is a genus of gram-negative bacteria comprising nearly 70 species and they are clinically human pathogens that can cause many infections. This study is mainly based on two species such as *Vibrio cholerae* O1 El Tor, which cause cholera disease and *Vibrio mimicus*, that cause gastroenteritis, open wounds infection, and septicemia. Therefore, this thesis aimed to study the effect of their virulence factors; melanin pigment, LuxO protein, ToxR protein, outer membrane proteins and vesicles on survival strategies; biofilm, rugose morphology and intracellular survival of *V. cholerae* and *V. mimicus* in *Acanthamoeba castellanii* using cell culture, viable cell count, gentamicin assay, vital staining, light-, fluorescent- and electron microscopy, microphotography, spectrophotometry, protein expression and isolation analysis, constructing internal in-frame gene deletion mutants and statistical analysis.

The results showed that *V. cholerae* mutant strain produced more melanin, showed increased UV resistance, expressed more toxin-coregulated pilus and cholera toxin and also increased colonization of infant mouse model compared to the wild-type *V. cholerae*. These findings suggest a possible role of melanin pigment formation in *V. cholerae* virulence factor expression.

V. mimicus had an enhanced growth in presence of *A. castellanii* and showed an intracellular behaviour in the amoeba. The bacteria were localized in the cytoplasm of amoeba trophozoites and the intracellular bacteria were viable for more than 2 weeks. Surprisingly, the *toxR* mutant of *V. cholerae* produced outer membrane protein T, significant biofilm and rugose colonies compared to the wild- type that produced OmpU, which showed decreased biofilm and did not form rugose colonies at 30°C. However, during the association with the amoebae it was observed that *A. castellanii* enhanced the survival of *V. cholerae* wild type compared to *toxR* mutant strain at 37°C. Interestingly, neither the wild- type nor *toxR* mutant strain could form rugose colonies in association with the amoebae. Therefore it shows that ToxR does seem to play some regulatory role in the OmpT/OmpU expression shift, the changes in biofilm, rugosity and survival with *A. castellanii*, suggesting a new role for this regulatory protein in the environments. Interestingly, outer membrane protein A (Omp A) suppressed the survival of alone cultivated wild-type *V. cholerae*, whereas the ompA mutant released more outer membrane vesicles and inhibited the viability of the amoebae. Co-cultivation of bacterial strains with *A. castellanii* enhanced the survival of both wild-type and ompA mutant but OmpA protein has no effect on attachment, engulfment and intracellular growth of *V. cholerae*.

In conclusion, this study based on the role of virulence factors on environmental survival strategies of *V. cholerae* and *V. mimcus* has demonstrated that melanin pigment plays an enhanced role in virulence and virulence factor expression of the bacteria such as resistance for UV- light, expression of toxin-coregulated pilus, cholera toxin and colonization ability.

V. mimicus shows an intracellular growth and survival in *A. castellanii* and neither LuxO regulator nor protease have role on the intracellular behaviour, which is reported for the first time.

The regulatory protein ToxR of *V. cholerae* suggests a new role in the expression of OmpT/OmpU and in the formation of biofilm, switching morphotypes from smooth to rugose colony and in association with the free living amoeba *A. castellanii*.

The outer membrane protein A of *V. cholerae* suppresses the survival of alone cultivated wild-type *V. cholerae* and has no effect on attachment, engulfment and intracellular growth of *V. cholerae* interacted with the amoebae, whereas *ompA* mutant released more outer membrane vesicles, which inhibits the viability of amoebae.

Keywords: *Vibrio cholerae*, *Vibrio mimicus*, melanin, *Acanthamoeba castellanii*, co-cultivation, gentamicin assay, outer membrane proteins, vesicles, rugose, biofilm, intracellular survival

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers:

I. **Valeru SP**, Rompikuntal PK, Ishikawa T, Vaitkevicius K, Sjöling A, Dolganov N, Zhu J, Schoolnik G, Wai SN. Role of melanin pigment in expression of Vibrio cholerae virulence factors. Infect Immun. 2009, 77(3):935-42

II. Abd H, **Valeru SP**, Sami SM, Saeed A, Raychaudhuri S, Sandström G. Interaction between Vibrio mimicus and Acanthamoeba castellanii. Environ Microbiol Rep. 2010, 2(1):166-171

III. **Valeru SP**, Wai SN, Saeed A, Sandström G, Abd H. ToxR of Vibrio cholerae affects biofilm, rugosity and survival with *Acanthamoeba castellanii*. BMC Res Notes. 2012, 16; 5:33.

IV. **Soni PriyaValeru**, Haifa Talal Alossimi, Salah Shanan, Amir Saeed, Gunnar Sandström and Hadi Abd. Outer membrane protein A suppresses survival of *Vibrio cholerae* and outer membrane vesicles inhibit viability of *Acanthamoeba castellanii* (Manuscript)

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CTX	Cholera toxin
TCP	Toxin co-regulated pilus
HGA	Homogentisic acid
VNBC	Viable but non-culturable
EPS	Exopolysaccharide
OmpT	Outer membrane protein T
OmpU	Outer membrane protein U
LPS	Lipopolysaccharide
OMVs	Outer membrane vesicles
FLA	free-living amoebae
UV	Ultra violet
LB	Luria-Bertani
Cb	Carbanecillin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Ttrichloroacetic acid
CRP	Cyclic AMP receptor protein
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Chromosomal deoxyribonucleic acid
RT	Real-time reverse transcription
PCR	Polymerase chain reaction
ATCC	American Type Culture Collection
PBS	Phosphate buffered saline

1 INTRODUCTION

Vibrio is a genus of Gram-negative bacteria that comprise nearly 70 species (1) including *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio mimicus*. Vibrio species are known to produce multiple extracellular cytotoxins and enzymes, which are associated with extensive tissue damage that may lead to the development of sepsis.

V. cholerae is classified into more than 200 serogroups based on the O antigenic structures(2). However, only serogroup O1 and O139 possess cholera toxin gene and produce cholera toxin causing epidemic and pandemic cholera that affects many millions annually (3-6).

The toxin-coregulated pilus, a type IV bundle-forming pilus required for intestinal colonization by *V. cholerae* is the other major virulence factor produced by *V. cholerae*. Expression of these virulence factors, cholera toxin and toxin-coregulated pilus are coregulated by a cascade of transcription factors (7-10).

V. cholerae species are straight or curved rods widely distributed in aquatic environments (11). Evidence suggests that *V. cholerae* is a component of the autochthonous flora of brackish water, estuaries, and salt marshes of coastal areas of the temperate zone, posing an ongoing hazard to public health (12, 13). Various *V. cholerae* O1 strains have become endemic in many regions in the world, including Australia and the Gulf Coast region of the US (4, 14). Cholera outbreaks are thought to have resulted from consumption of raw, undercooked, contaminated, or re-contaminated seafood since *V. cholerae* is transmitted primarily by the faecal-oral route, indirectly through contaminated water supplies (13, 15-18).

Melanization has been considered to be important in microbial pathogenesis because of its association with virulence in many microorganisms (19, 20). Melanin appears to contribute to virulence by reducing the susceptibility of melanized microbes to host defense mechanisms. Although melanin pigments are not considered essential for the growth of microorganisms, they can increase the survival of microorganisms by various means like protecting DNA and other molecules from UV light, enhance virulence, inhibit biofilm degradation, protect enzymes from proteases, and protect microorganisms from hydrolytic enzymes, and they may even act as proton and nutrient sinks in biofilm (21, 22). Melanization, and its consequences for mammalian virulence, has been most extensively studied in *Cryptococcus neoformans* (20). The pathway of tyrosine metabolism, which could lead to pigment formation, involves two branches: melanin formation via the hydroxyphenylalanine intermediate, which requires tyrosinase activity, and

tyrosine catabolism involving homogentisic acid (HGA) formation and its further conversion to maleylacetoacetic acid (23). HGA is the main *p*-diphenolic intermediate of the normal L-tyrosine catabolism pathway in animals and bacteria. HGA is known to autooxidize, leading to the formation of reddish-brown pigment called pyomelanin, HG-melanin, or ochronotic pigment. In vitro studies have shown that HGA auto-oxidation is catalyzed by Mn2 and involves H_2O_2 formation during the reaction (23-25). Several bacterial species are known to produce pigment via the HGA intermediate, including *Shewanella colwelliana*, *Hyphomonas* species, and *Legionella pneumophila* (25, 26). Most of the *V. cholerae* strains do not form any detectable pigment under normal experimental growth conditions normally used, however some pigmented mutants were detected after *N*methyl-*N*-nitro-*N*-nitrosoguanidine induced mutagenesis (19, 27). The mutated gene(s) in such mutants remains unverified. It has been shown that pigment production in *V. cholerae* can be induced in response to stress, particularly hyperosmotic shock and elevated temperatures (7, 28-30). The pigment formation is initiated in late-exponential to postexponential growth phase of the bacteria (7, 29). *V. cholerae* is a member of a relatively large group of environmental bacteria that produce melanins, including species of *Aeromonas*, *Burkholderia*, *Caulobacter*, *Mycobacterium*, *Proteus*, *Pseudomonas*, *Serratia*, and *Legionella* (28). Melanins are also broadly distributed among eukaryotic microorganisms, including fungi and protozoa (20, 31). The HGA branch is believed to be the only catabolic pigment-producing branch present in *V. cholerae* (25, 32).

Vibrio mimicus shares similar properties with *V. cholerae* such as existence of similar virulence associated genes, namely cholera toxin as well as toxin co-regulated pilus genes (33) and both species possess LuxO protein that regulates protease activity(34). *V. cholerae* adopts several survival strategies in aquatic environments. The bacterium can survive as free-living or in association with phytoplankton/zooplankton, crustaceans, and molluscs in coastal and estuarine environments (35). *V. cholerae* forms biofilms on biotic and abiotic surfaces, thereby protecting themselves from toxic compounds, such as antibiotics, thermal stress, and predation (36) with this exopolymer barrier(37) . The bacterium has been described to switch between the smooth and rugose colony morphotypes contributing to its environmental survival. The exopolysaccharide (EPS) materials of rugose colony-forming *V. cholerae* strains absent from smooth colony *V.cholerae* were described as a heavy, fibrous, electron-dense, ferretin-stained layer surrounding the cells(38). The cell surface of EPS materials confers a rugose colony morphology and resistance to osmotic and oxidative stresses. The regulation of EPS synthesis in bacteria is complex and involves multiple systems utilizing both positive and negative regulation (38).

Main virulence factors of *V. cholerae* such as CTX and TCP are coregulated by a transcriptional regulator ToxR. ToxR is a transmembrane DNA binding protein, however, independently of the transcriptional activators TcpP and ToxT, it modulates expression of two outer membrane proteins OmpU and OmpT. Transcription of ompU is induced by ToxR, whereas transcription of ompT is repressed by ToxR (39, 40).

Bacteria respond to changes in their environment by global changes in transcription. The regulation of certain transcriptional membrane proteins controls the expression of outer membrane porins. *V. cholerae* outer membrane is composed of a limited number of protein moieties and lipopolysaccharide (LPS) (41, 42). However, it was found that OmpA protein of *V. cholerae* shares 47.8% similarity to *Escherichia coli* OmpA protein. OmpA is a β-barrel protein in the membrane and is highly conserved among Gram-negative bacteria (43). It is expressed to very high levels and it is tightly regulated at the post-transcriptional level. It can function as an adhesin and invasin, participate in biofilm formation, act as both an immune target and evasin, and serves as a receptor for several bacteriophages (44, 45). It has been shown that *E. coli* utilized the OmpA protein for adhesion to HeLa epithelial cells and Caco-2 colonic epithelial cells (46).

Outer membrane vesicles (OMVs) are produced by most Gram-negative bacteria including Vibrio species (47). OMVs contain outer membrane proteins, lipopolysaccharides, phospholipids and as vesicles are being released from the surface, they entrap some of the underlying periplasm. They can deliver toxins and other virulence factors to the host at relatively high concentrations without requiring close contact between the bacterial and target human cells, and they are believed to represent a key factor in effecting an inflammatory response in the host toward bacterial pathogens (48-53).

Defects in protein either linking the outer membrane to the peptidoglycan layer or involved in a structural network between the inner, outer membranes and the peptidoglycan layer result in the shedding of large amounts of OMVs (51), whereas outer membrane proteins play a major role in the adherence to mucosal membrane in the small intestine and possible protective antigens (54).

Vibrio species and free-living amoebae (FLA) are present in aquatic environments, including drinking water (55-57). *Acanthamoeba* is a genus of FLA, which are environmental eukaryotic cells distributed worldwide in nature (58, 59) and they are found to support bacterial growth and survival (58). Interestingly, *V. cholerae* O1, O139 and *V. mimicus* have grown and survived inside *Acanthamoeba* species (60-64). Vibrio species utilize different survival strategies and possess different virulence factors such as melanin pigment, outer membrane protein A,

regulatory proteins LuxO and ToxR in addition to outer membrane proteins A. This thesis aimed to study the role of melanin pigment, LuxO, ToxR, and outer membrane vesicles on survival strategies such as biofilm, rugose survival form and intracellular survival of *V. cholerae* and *V. mimicus* in *A. castellanii* using cell culture, viable cell count, gentamicin assay, vital staining, light-, fluorescent- and electron microscopy, microphotography, spectrophotometry, internal in-frame gene deletion, protein expression and isolation analysis and statistical analysis.

2 AIMS OF THE PROJECT

The overall aim of this thesis work is to study the effect of virulence factors on the survival strategies of *Vibrio* species by using *Acanthamoeba castellanii* as a host model.

Specific aims:

Paper I

- To study the role and pathogenicity of pigment over- producing *V. cholerae*

Paper II

- To study the interaction of *Acanthamoeba castellanii* with *V. mimicus*

Paper III

- To study the effect of ToxR regulatory protein in the expression of OmpU and OmpT, rugosity and biofilm formation as well as association with *A. castellanii*

Paper IV

- To study the role and influence of OmpA and OMVs towards the survival of *V. cholerae* alone and its interaction with *A. castellanii*

3 MATERIALS AND METHODS

Following are the summarized description of materials and methods used in this thesis, however specific details of the performed experiments can be found in the respective papers.

3.1 BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Strain	**Description**	**Reference**
V. cholerae **(paper I, III, IV)**		
A1552	Wild type *V. cholerae*, smooth ,O1 El Tor Inaba	(65)
ΔtoxR	Transcriptional deletion mutant	This study
ΔompA	Inframe deletion mutant	(44)
SNW 29 (*hmgA::km*)	Pigment producing mutant	This study
SNW 30 (*ΔrpoS*)	Inframe deletion mutant	This study
SNW 31 (*ΔrposhmgA::km*)	Double mutant	This study
ΔVC1348	Deletion mutant	This study
ΔVC1349	Deletion mutant	This study
V. mimicus **(paper II)**		
CS-5	Wild type *V. mimicus* smooth	(34)
LODC-5 (*ΔluxO*)	Disruptant mutant	(34)

Escherichia coli strains DH5α (Φ80d*lacZ*ΔM15 *recA1gyrA96 thi-1 hsdR*17 (r_k^- m_k^+) *supE44 relA1deoR*Δ *(lacZYA-argF) U169*) (67) and S17-1λ*pir (hsdR recA pro RP4-2 Tc::Mu; Km::Tn7 (λpir)* (69) were used for standard DNA manipulations and as donors in mating in transposon mutagenesis. Suicide plasmids pCVD442 (oriR6K *mobRP4 sacB* $Ap^{r)}$ (70) and Puc18 (Ap^R lacZaovi colE1) (stratagene) are used for conjugal transfer to rifampin-resistant *V. cholerae* O1 El Tor A1552.

3.2 CULTURE MEDIA AND GROWTH CONDITIONS

All bacterial strains were grown at 37º C using LB medium (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl). In paper I, LB agar plates, and several defined media were used for preliminary studies of growth and pigment production of the mutant and the wild-type strains of *V. cholerae.* In paper II, all strains were grown in LB with appropriate antibiotic selection (50 µg/mL carbenicillin or 50 µg/mL streptomycin) and two different temperatures (25º C and 37º C) were used for co-cultivation studies. In Paper III and IV, all strains were grown in LB media at 37º C. Blood agar plates were used for colony count (paper II, III, IV). *A. castellanii* was grown without shaking at 30º C to a final concentration of 10^6 cfu /ml in ATCC medium no. 712.

3.3 GENOME DATABASE SEARCH

Genome database accession number searches were done for the complete whole-genome sequence of *V. cholerae* O1 biovar El Tor strain N16961 chromosomes I and II at the NCBI website. (www.ncbi.nlm.nih.gov).

3.4 TRANSPOSON MUTATION AND COMPLEMENTATION (PAPER I)

Tn5 mutagenesis of *V. cholerae* O1 El Tor, strain A1552, was performed by conjugation. A total of 30,000 exconjugants were screened on plates to identify mutants exhibiting a pigment production phenotype. Chromosomal DNA was isolated from the *V. cholerae* transposon insertion mutants using the Marmur procedures (66).The DNA fragments extracted were ligated using ligase enzyme (Takara Bio Inc, Ostu, Shiga, Japan) into the cloning vector pUC18 that had been digested with the EcoRI enzyme. The pool of plasmids was introduced by transformation into *E. coli* strain DH5α by electroporation, followed by the selection of resistant transformants on plates containing 30 µg/ml kanamycin (Sigma, Solkraftsvägen 14c, Stockholm, Sweden) and 100 µg/ml Cb (Sigma, Solkraftsvägen 14c, Stockholm, Sweden) resistant transformants. The DNA fragment containing the oxidoreductase gene was obtained after amplification by PCR using WT *V. cholerae* chromosomal DNA as a template. The PCR product was purified and isolated. The islolated *hmgA* plasmid clone (pSNW501) was electroporated into WT and SNW29 mutant of *V. cholerae.* The vector pCR2.1 without an insert was similarly introduced into the WT and mutant strains as a negative control.

3.5 MEASUREMENT OF PIGMENT PRODUCTION AND UV IRRADIATION TEST (PAPER I)

Bacterial strains were grown in LB medium at 37° C with shaking conditions overnight. One hundred microliters of overnight bacterial culture was inoculated into 20 ml LB medium in the presence or absence of 5 mM L-tyrosine (Sigma, Sigma, Solkraftsvägen 14c, Stockholm, Sweden). Cultures were grown at 37° C under shaking conditions overnight. The culture supernatants were removed by centrifugation at 15,000x *g* for 10 min and filtered through a 0.2-µm Millipore filter (Millipore AB, Abingdon, Oxfordshire, UK). The pigmentation of the culture supernatants was measured by the OD 400. The data represent the results of three independently performed experiments. Bacterial strains were grown in LB medium with aeration by shaking at 37° C for 24 h and the bacterial suspensions were transferred into a petri dish and irradiated with UV doses of 0, 100, 200, and 300 µJ/m^2. All were performed three to five times, and an average was calculated from the analyses of surviving bacterial cells after serial dilutions of bacterial suspensions were plated on LB agar incubated in the dark at 37° C overnight.

3.6 SDS-PAGE (PAPER I AND III) WESTERN BLOTTING AND TCPA STABILITY TEST (PAPER I)

For TcpA and CTX detection, *V. cholerae* strains were grown as described previously (67)**.** Bacteria were harvested by centrifugation at 10,000x*g* for 10 min at 4° C. The resulting pellet was resuspended in 0.20 volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2-mercaptoethanol and used for the detection of TcpA. The culture supernatant fluid was precipitated with 10% trichloroacetic acid (TCA). Proteins with known molecular masses (Fermentas, Pittsburgh, United States) were used as molecular mass markers. The gels were fixed with methanol and glacial acetic acid. The proteins were visualized by Coomassie blue staining. Western blot analyses were performed as described previously (68), Anti-TcpA polyclonal antiserum (a gift from R. Taylor (69) and anti-CTX A and B subunit antiserum (Sigma, Solkraftsvägen 14c, Stockholm, Sweden) were used for detection of TcpA in whole-cell extracts and CTX in culture supernatants, respectively. The anti-CRP polyclonal antiserum against *E. coli* cyclic AMP receptor protein (CRP**)** (47, 70) was used for the internal control of cytoplasmic protein when the TcpA immunoblot analysis was performed. Expression of outer membrane proteins in paper II was also performed by using 13% SDS-PAGE. Intracellular and surface-bound TcpA stability was determined by using a

technique described previously (69). The protein stability was monitored after protein synthesis had been inhibited by the addition of 25 µg/ml chloramphenicol to bacterial cultures grown to 40 Klett units in LB medium at 37° C. Samples to be analyzed by Western blotting were removed at the indicated time points: 0, 5, 10, 15, 30, 45, and 60 min after the addition of chloramphenicol.

3.7 QRT-PCR AND MICROARRAY EXPERIMENTS

RNA was prepared using Trizol according to the manufacturer's instructions (Invitrogen, Fountain Drive Inchinnan Business Park Paisley PA4 9RF, UK) and the concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Silverside Bancroft, Wilmington DE 100, USA) cDNA was prepared from 200 ng RNA from each sample and Real-time reverse transcription (RT)-PCR was performed using specific primers. Each reaction mixture contained 300 nM primers, 100 ng template, and reference dye. Three independent samples were tested in triplicate. For each sample, the mean cycle threshold of the test transcript was normalized to that of tmRNA. For microarray experiments, bacteria were cultured statically for 4 h in AKI medium (67) at 37° C and then shifted to aerobic growth for 6 h using shaken culture flasks (67). Triplicate samples were taken every hour for the duration of the experiment (10 h). RNA was isolated, treated with DNase I, and cleaned with an RNease kit (Qiagen, Valencia, CA, USA) Labeling of cDNA and microarray hybridizations were performed as described previously (69). RNA from bacteria exponentially grown in LB (OD 600) was used as a reference. Microarray data analysis was done as described previously (71).

3.8 INFANT MOUSE COMPETITION ASSAY

Approximately 10^5 bacterial strains cells were inoculated intragastrically into 6-day-old CD-1 mice (Charles River Laboratories, Ballardvale street, Wilmington, USA) The mice were euthanized after 20 h, and the bacteria colonizing the intestines were quantified as described previously (72).

3.9 CONSTRUCTION OF THE INTERNAL IN-FRAME DELETION MUTANT (PAPER I, III AND IV)

We used a double cross over procedure to introduce a deletion at the chromosomal locus. A plasmid pCVD442 was used as a suicidal vector. In the first step two different asymmetric pol-

ymerase chain reactions (PCR) amplified with primers to generate fragments upstream and downstream of the sequences targeted for deletion. In the second step, the upstream and downstream fragments were annealed at their overlapping region and amplified by PCR as a single fragment, using the outer primers. The PCR products were phenol-chloroform extracted,

ethanol precipitated, washed with 70% ethanol, vacuum dried, resuspended in 50 mL of Xba-I restriction buffer containing 40 U of XbaI restriction enzyme, and digested overnight at 37°C. The DNA fragments were gel purified, ligated into XbaI digested and alkaline phosphatase-treated pCVD442 and then, electroporated into E. coli SM10λpir or S17-1 λ*pir*. The mutant constructs werechecked by PCR and sequencing. The confirmed deletion constructs were introduced into *V. cholerae* by conjugation, screened on 15% sucrose containing plates, and the carbenicillin sensitive clones were assessed for double cross-over recombination. The recombinant colonies were checked by PCR. (For the primers used in this thesis, please refer to the related articles).

3.10 BIOFILM ANALYSIS AND COLONY MORPHOLOGY (PAPER III)

Bacterial strains were grown in LB broth at 37º C in shaking incubator until the absorbance reached 0.6 units at 600 nm. 5 mL of 1:100 dilution of this suspension was transferred into triplicated glass tubes and incubated for 18 h at 30° C. Representative biofilm formed by the strains was visualised photographically and the tubes were incubated with 1% crystal violet stain for 15 min. The biofilm-associated crystal violet in the tubes was suspended with 95% ethanol and measured by spectrophotometry at 570 nm to quantify the biofilm formation. For colony morphology assay, bacterial strains were grown on LB agar plates for 1 day at 30° C and left at room temperature until appearance of rugose colonies. Percentage of rugose colonies was estimated and representative smooth as well as rugose colonies were visualized photographically after day 6.

3.11 CO-CULTIVATION ASSAY (PAPER II, III, IV)

Co-cultivation assay was based on a method presented previously (64). Co-cultivations of bacterial strains with *A. castellanii* were incubated in culture flasks (75 cm) filled with 50 mL ATCC medium 712 containing *A. castellanii* at a concentration of 2×10^5 CFU/mL and the particular *V. cholerae* species at a concentration of 2×10^6 CFU/mL. Control flasks containing bacteria or amoebae only were prepared in the same way and with the same initial concentration

as the co-culture flasks. All flasks were triplicates and incubated 30º C in general for paper II we used two different temperatures (25° C and 37° C). Samples were taken and plated on blood agar plates regularly to study growth and survival of bacterial strains.

3.12 ENCYSTATION OF *A. CASTELLANII* (PAPER II)

Encystation of *A. castellanii* harbouring intracellular *V. Mimicus* (paper I1) was performed to show that viability of the amoebae is not affected by the intracellular *V. mimicus* because the encystation is a vital process of the amoebic life cycle. To examine the role of *Acanthamoeba* cysts to protect the intracellular bacteria from high level of gentamicin, *A. castellanii* cells were cultivated with *V. mimicus* CS-5 in ATCC 712 medium for 1 day, treated with 1000 mg/ml gentamicin to extracellular bacteria, recultivated in PBS and after the encystation process again treated with gentamicin. Viable counts were performed for the amoebae and intracellular *V. mimicus*.

3.13 BACTERIAL UPTAKE, INTRACELLULAR GROWTH SURVIVAL AND LOCALIZATION (PAPER II AND IV)

Samples were taken from co-culture flasks and viable counts of engulfed, intracellular survival of bacterial strains were investigated. A series of 10-fold dilutions of the sample was prepared and spread on blood agar plates. All plates were incubated at 37º C for 24 h. Gentamicin and sodium deoxycholate treatment were utilized to kill extracellular bacteria and to permeabilize amoeba cells in order to release the intracellular bacteria. Beside the viable count assay, transmission electron microscopy was used to disclose intracellular localization of *V. mimicus* in *A. castellanii* (paper IV). Samples of amoebae in absence and presence of *V. mimicus* were cut into ultrathin sections to differentiate between intracellular and extracellular *V. mimicus*. According to the ultra-thin sections technique intracellular bacteria would be found inside the amoeba cells while extracellular bacteria would be found attached to the outside of amoeba cells.

3.14 BACTERIAL ADHERENCE ASSAY (PAPER IV)

Co-cultures of bacterial strains incubated with *A. castellanii* at 30º C and samples were withdrawn after 1 h to determine the percentage of bacteria adhered to the amoeba cells by

dividing the number of amoebae with adhered bacteria by the total number of amoebae with and without adhered bacteria, multiplied by 100.

3.15 ISOLATION OF OUTER MEMBRANE VESICLES AND ESTIMATION OF PROTEIN CONCENTRATION (PAPER IV)

OMVs were isolated by ultracentrifugation as described earlier (73)**.** *V. cholerae* strains were grown in broth culture to late exponential phase. Broth cultures were then centrifuged at 8,000x *g* (30 min, 4° C) in a JA-25.50 rotor (Beckman Instruments Inc, Fullerton, California, USA) Filtered (0.22 µm; Millipore) supernatants were centrifuged at 85,000x *g* (2 h, 4° C) in a 70 Ti rotor (Beckman Instruments Inc, Fullerton, California, USA) to collect OMVs. Total protein concentration was measured by the Bradford assay (Bio-Rad, Solna, Stockholm, Sweden). Effect of outer membrane vesicles on viability of *A. castellanii* was examined by incubation 50 µl amoeba cells suspension of $2x10^6$ cells/ ml with 50 µl OMVs preparation from each bacterial strain or with 50 µl PBS for controls. Triplicate experiments were performed and the viability of amoebae examined after 2 hours by viable count utilizing erythromycin stain.

3.16 STATISTICAL ANALYSIS

Student´s t-test were used to examine for significant differences in growth between alone and co-cultivated amoebae as well as bacteria.

3.17 METHODOLOGICAL CONSIDERATIONS

In microbiological work, technical problems vary between microorganism's genera or species, and even between strains within a species. *A. castellanii* is cultivated easily in the ATCC media, easy to count and stable in the media used. The technical problem was faced in preparatory work related to the concentration of gentamicin to kill all the extracellular bacteria required methodological development. Although similar methods were used in the publications, the reproducibility of the methods allowed improvement of the methods used.

4 RESULTS

Results of this thesis high lightened the role of melanin pigment, ToxR and outer membrane proteins of *V. cholerae* and LuxO protein of *V. mimics* virulence and survival strategies such as resistance to UV-light, formation of biofilm and rugose colonies and intracellular survival in *A. castellanii.*

4.1 ROLE OF MELANIN PIGMENT IN EXPRESSION OF *VIBRIO CHOLERAE* VIRULENCE FACTORS

4.1.1 Identification and analysis of a pigment-producing *V. cholerae* mutant.

Among the 30,000 transposon mutants, two pigmented colonies were identified in a screening on solid medium. One mutant isolate, denoted SNW28, was chosen for further analysis. Upon growth in liquid medium, the culture of the mutant turned distinctly brown Fig. 1A. Mutated gene locus in the SNW28 isolate was mapped and found that the transposon insertion had occurred after nucleotide 681 in an open reading frame corresponding to the locus denoted VC1345 (accession number, AE003852.1; GeneID, 2614799) in the *V. cholerae* genome Fig.1B. The second brown-pigmented mutant was found to have a transposon insertion in the VC1345 gene. By using *trans*-complementation test it was shown that the *hmgA*$^{+}$ clone complemented the mutation, and in its presence, the pigment production was lost Fig. 1C. We also observed that the melanin pigment-producing mutant strain SNW29 was more resistant to UV irradiation than the WT *V. cholerae* strain Fig. 2.

Fig. 3 represents the catabolic pathway of tyrosine metabolism and representative genetic loci.

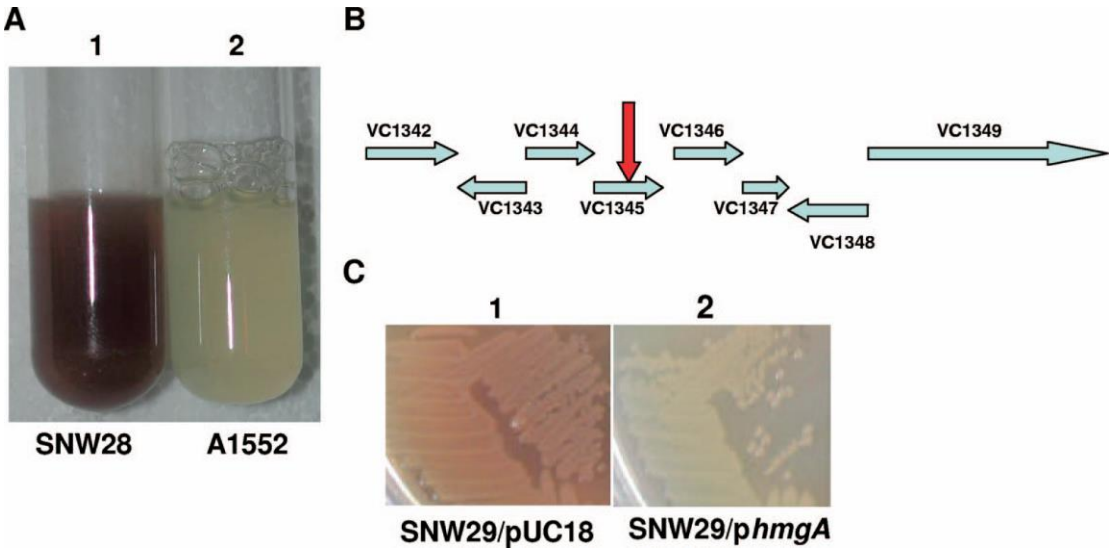

FIG.1. (A) Pigment production after bacterial growth in LB liquid medium at 37° C with shaking. (B) Genetic map of the mini-Tn*5*–Km2cassette insertion in the brown-pigment overproducing *V. cholerae* mutant at chromosome position VC1345 (accession number, E003852.1; GeneID, 2614799) (arrow). (C) Complementation analysis of the brown-pigment-producing mutant.

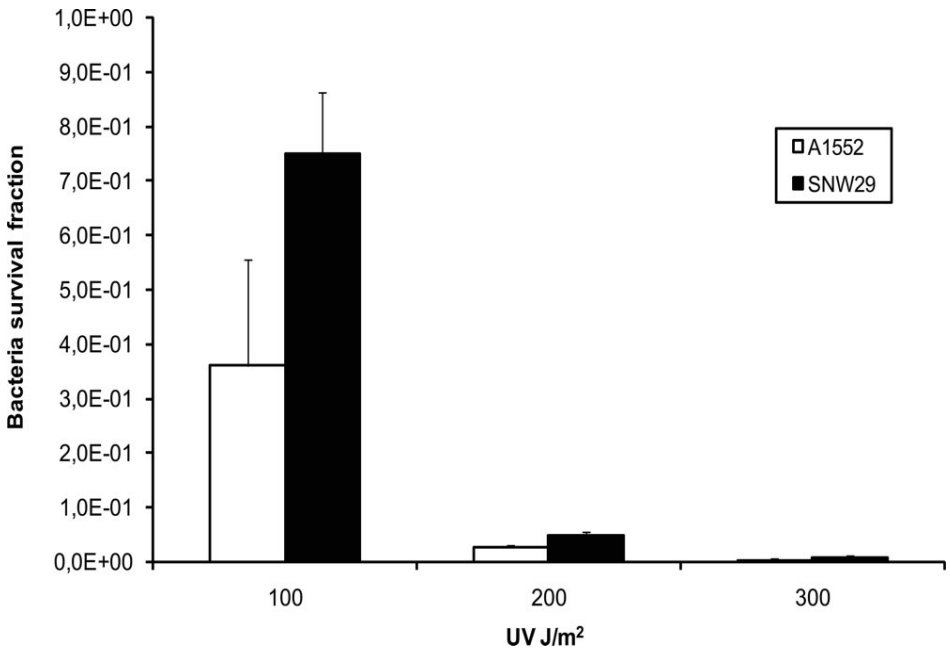

FIG. 2. UV sensitivity test of *V. cholerae* A1552 (WT) and SNW29.

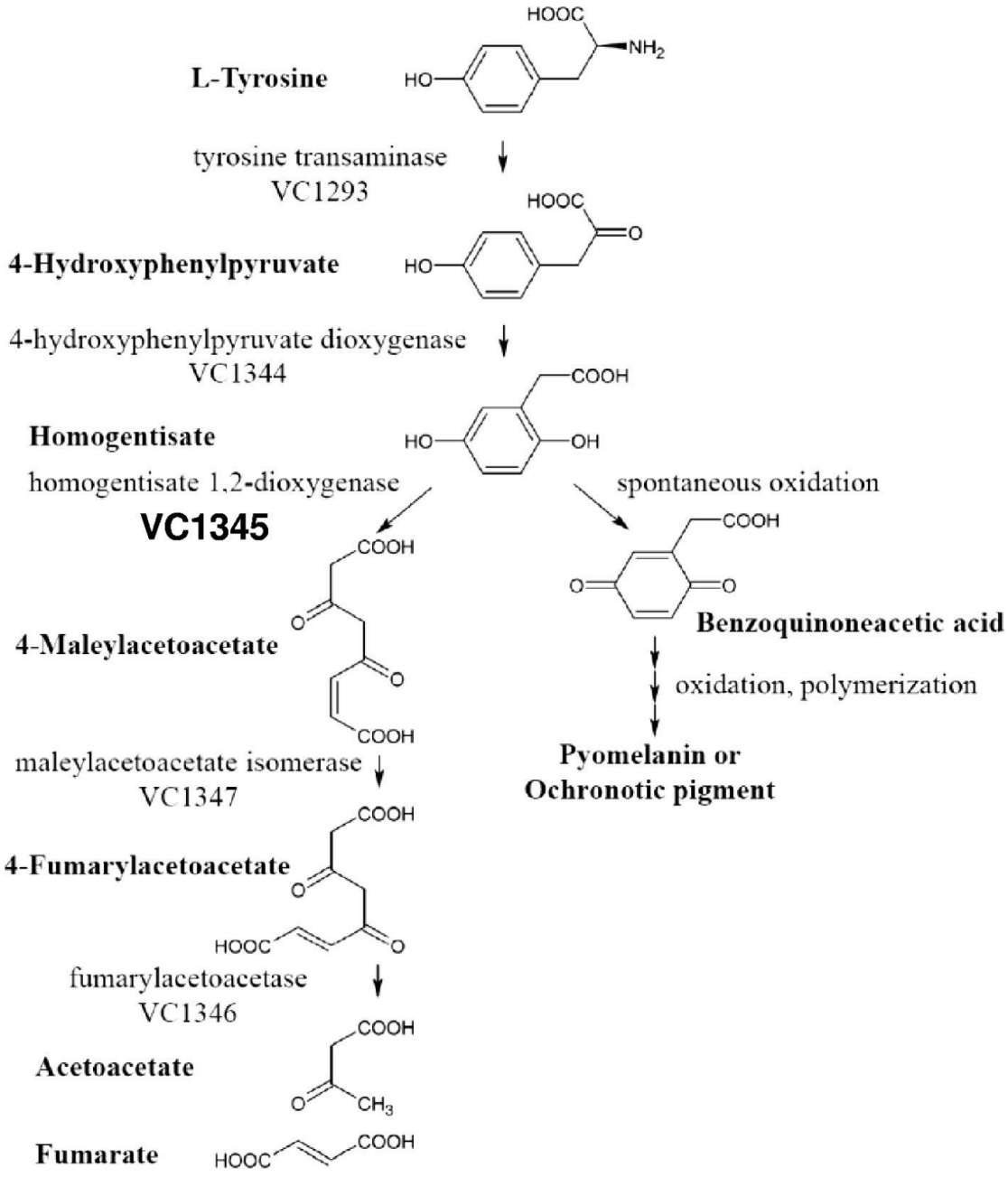

FIG. 3. Catabolic pathway of tyrosine metabolism and representative genetic loci. Predicted enzymes encoded by the respective genetic loci in *V. cholerae* are as follows: VC1344, 4-hydroxyphenylpyruvate dioxygenase; VC1345, homogentisate 1,2-dioxygenase; VC1347, maleylacetoacetate isomerase; VC1346, fumarylacetoacetase. The proposed pathway is based in part on the pathway described for *Streptomyces avermitilis* (5).

4.1.1 Analysis of growth phase-dependent expression of *hmgA* gene

Growth phase-dependent expression of the *hmgA* gene and the neighboring genes such as VC1344 and VC1346 to VC1349 was studied by using microarray expression profiling assay at transcriptional level as shown in Fig. 4. We observed that the expression of the VC1344-to-VC1349 gene cluster was influenced by the growth phase, when the cells had reached the stationary phase Fig. 4. Two of the neighboring genes, VC1348 and VC1349, annotated to encode a putative response regulator and a sensor kinase, respectively, were observed to have the highest expression levels in the stationary phase Fig. 4.

In order to test if VC1348 and/or VC1349 mutants would affect expression of the VC1344-VC1345 operon, we introduced deletion mutations into each gene and performed qRT-PCR analyses as summarized in Fig. 5. It was observed that mutation in either VC1348 or VC1349 caused reduced expression levels of both VC1344 and VC1345. The levels of transcription of the VC1344 and *hmgA* genes in WT *V. cholerae* strain A1552 and in SNW30 were compared by using qRT-PCR. As it was shown in the Fig 5, the expression of both the *hmgA* and VC1344 genes were 8-fold reduced in the *rpoS* mutant of *V. cholerae* strain A1552. As shown in Fig. 6, we tested the levels of pigment production in WT *V. cholerae*, the *hmgA* mutant (SNW29), the *rpoS* mutant (SNW30), and the *hmgA rpoS* double mutant (SNW31) in the absence or presence of exogenously added tyrosine in the culture medium and a slight increase in the production of pigment was observed in the *hmgA rpoS* double mutant (SNW31) grown in the presence of tyrosine compared with the *hmgA* single mutant (SNW29). However, there were no differences observed in pigment production between the WT and the *rpoS* single mutant (SNW30) strain when grown either in the absence or in the presence of tyrosine.

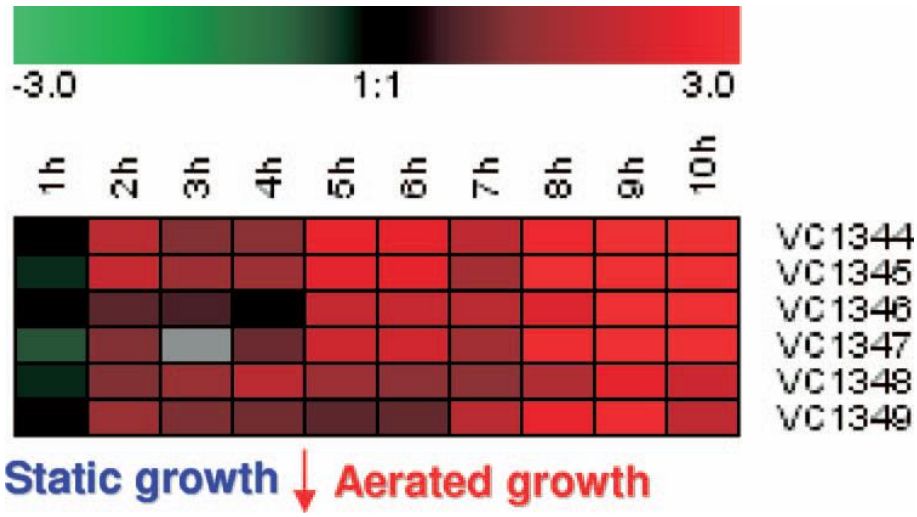

FIG. 4. Expression of the VC1344-VC1349 gene cluster during 10 h of bacterial growth under AKI conditions. Gene expression of WT A1552 was analyzed using RNA from exponentially grown bacteria in LB culture as a reference. The expression ratios are represented by shades of color according to the log2 (induction) scale shown

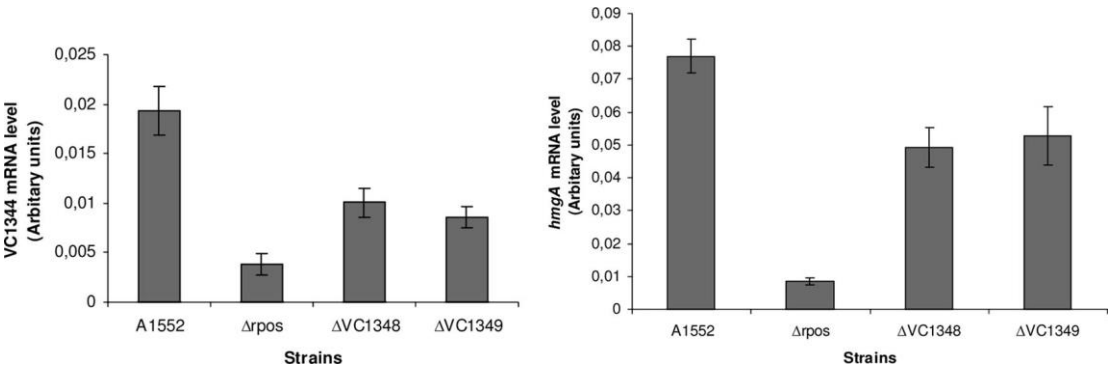

FIG. 5. Analyses of VC1344 and VC1345 (*hmgA*) gene expression by qRT-PCR as described in Materials and Methods. The data represent the results of three independently performed experiments. The error bars indicate the standard deviations from three experiments.

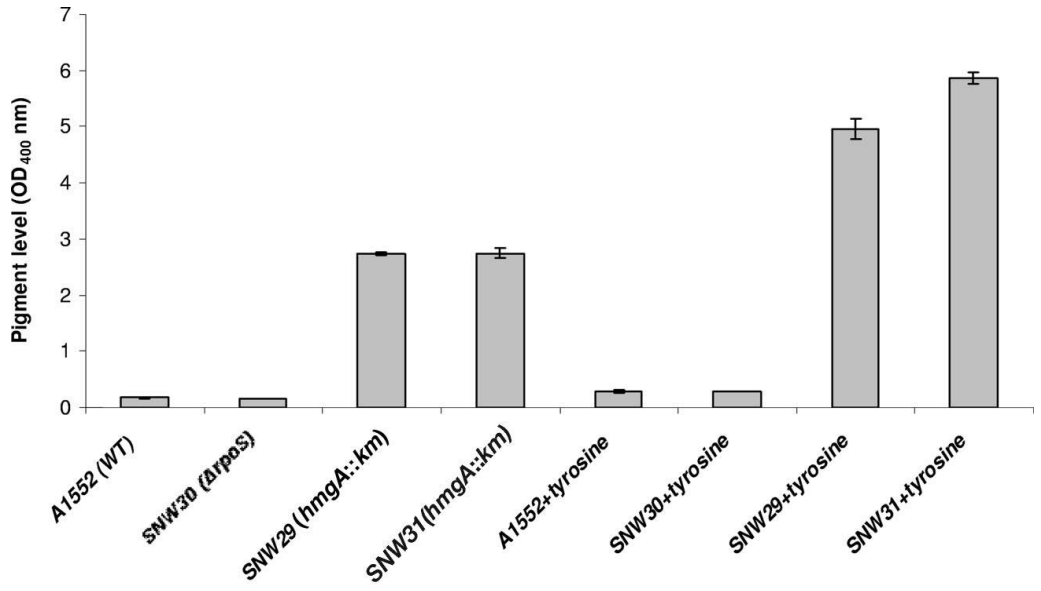

FIG. 6. Levels of pigment production in the WT *V. cholerae* strain A1552, the *rpoS* mutant (SNW30), the *hgmA* mutant (SNW29), and the *hgmA rpoS* mutant (SNW31) with and without tyrosine supplement in growth medium. The error bars show the standard variations of three experiments

4.1.2 Effect on *V. cholerae* virulence factors expression of the *hmgA*::mini-Tn*5* mutation.

We found an elevated level of secreted CTX in the *hmgA*::mini-Tn*5* mutant in comparison with the WT strain, A1552 as shown in Fig. 7A. In order to further investigate the putative link between pigment formation and virulence factor expression, the level of the toxin-coregulated pilus, Tcp, was analyzed and as an internal control, the CRP transcriptional factor, in strains A1552 and SNW29. As shown in Fig. 7B, there was a clear increase in the level of TcpA protein, the main Tcp pilus subunit, as a result of the *hmgA* gene mutation. Relative stability of TcpA in the mutant and parental strains did not reveal much difference in TcpA stability Fig. 8A and B. To test if, for example, H2O2 per se could induce higher expression of CTX and the toxin-coregulated pilus, we monitored the level of TcpA after incubation of *V. cholerae* strain A1552 in the presence of different H_2O_2 concentrations. As shown in Fig. 8C, the TcpA level was increased by increasing concentration of H2O2.

Using colonization of the infant mouse intestine as an infection model, the *hmgA*::mini-Tn*5* mutant was examined, and its colonization efficiency was compared to that of the parental strain, A1552. As it was shown in the Fig 9, colonization by the mutant strain was

about fivefold higher than that by the WT strain. As colonization efficiency is also dependent on bacterial growth, the growth rates of strains SNW29 and A1552 in LB were determined, and we observed no growth rate difference between the WT and the mutant (data not shown). Taken together, the results suggest that the increased colonization of strain SNW29 in the infant mouse model might be due to the increased expression of TcpA.

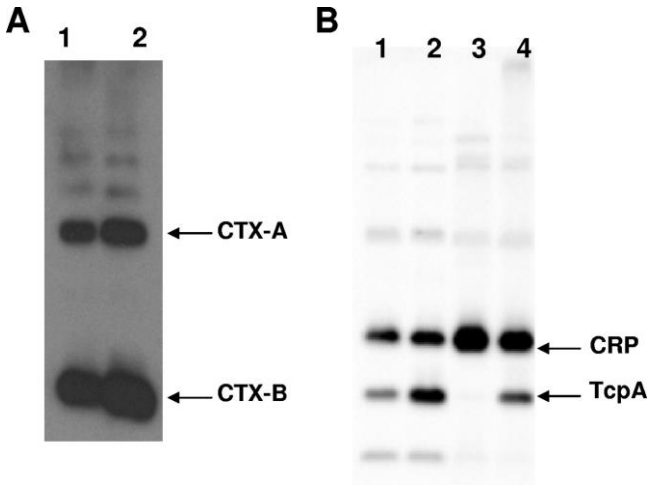

FIG. 7. (A) Immunoblot analyses of CTX levels in *V. cholerae* WT A1552 (lane 1) and SNW29, a hyperpigmented strain (lane 2), with anti-CTX polyclonal antiserum. (B) TcpA production in A1552 (WT) and SNW29 (hyperpigmented) strains. The samples in lane 1 (A1552) and lane 2 (SNW29) were from bacteria grown in yeast extract-peptone (YEP) medium at 37°C under static culture conditions. The samples in lane 3 (A1552) and lane 4 (SNW29) were from bacteria grown in YEP medium at 37°C with shaking. The CRP protein was detected as an internal control for cytoplasmic protein. The arrows show the positions of the TcpA and CRP proteins. A total of 3 μg of protein was loaded in each lane.

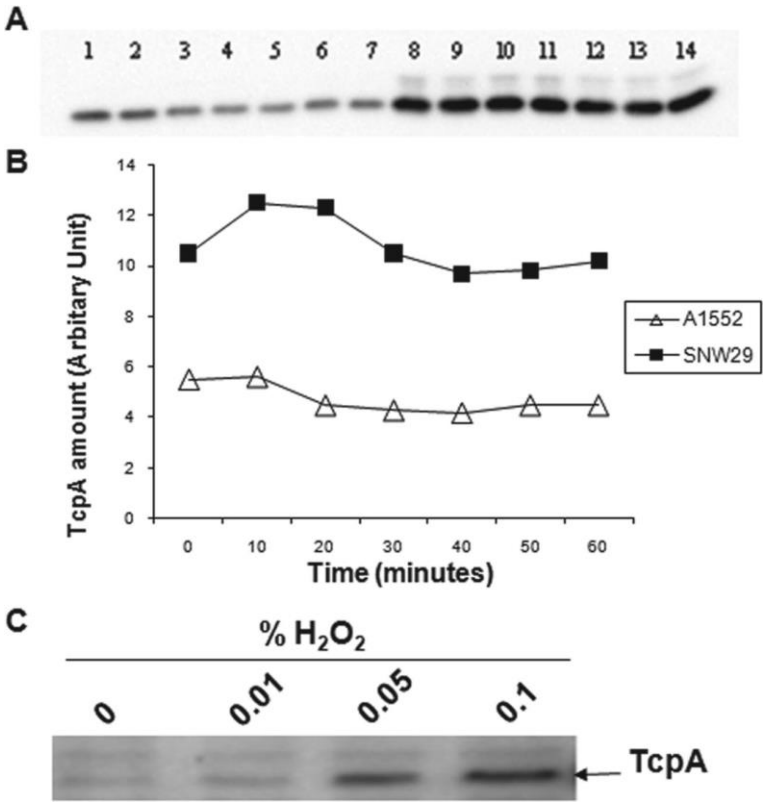

FIG. 8. Comparison of TcpA protein stabilitiesy in WT strain A1552 and the mutant SNW29. (A) Immunoblot analysis using anti-TcpA polyclonal antiserum. Lanes 1 to 7, A1552; lanes 8 to 14, SNW29. The times after addition of chloramphenicol were as follows: lanes 1 and 8, 0 min; lanes 2 and 9, 5 min; lanes 3 and 10, 10 min; lanes 4 and 11, 15 min; lanes 5 and 12, 30 min; lanes 6 and 13, 45 min; lanes 7 and 14, 60 min. (B) Quantitative analysis of TcpA levels using the Quantity One program (Bio-Rad). (C) Effect of H2O2 on TcpA expression in *V. cholerae* strain A1552. Shown is immunoblot analysis using anti-TcpA antiserum. The bacteria were grown in the presence of different concentrations of H_2O_2.

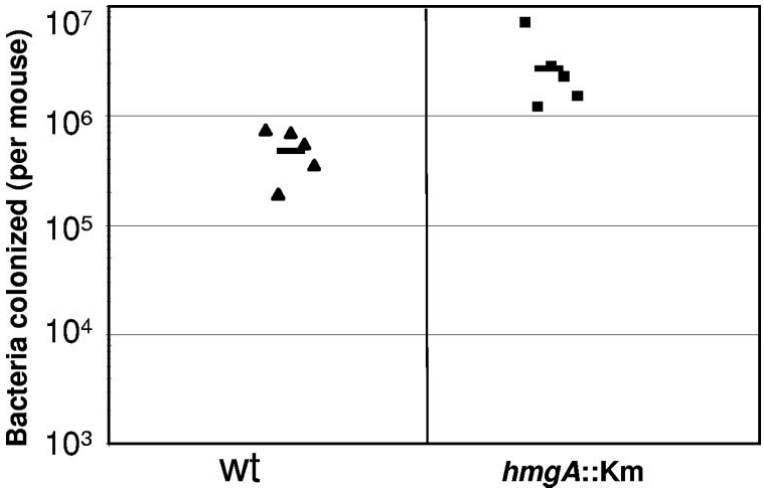

FIG. 9. Infant mouse colonization assay using strains A1552 (wt) and SNW29 (*hgmA*::Km) as described in Materials and Methods.

4.2 INTERACTION BETWEEN *VIBRIO MIMICUS* AND *ACANTHAMOEBA CASTELLANII*

4.2.1 Effect of *V. mimicus* on the growth of *A. castellanii*

To study the ability of amoebae to grow alone and during co-cultivation, cell counts of viable *A. castellanii* in the absence and the presence of a wild and *luxO* mutant strain of *V. mimicus* were performed.

We found eightfold respective sevenfold increase of amoebae in the presence of wild or mutant strain after 14 days Fig. 10. Based on χ^2 test no statistically significant difference was found in the growth of *A. castellanii* in the presence or absence of *V. mimicus* CS-5 or its mutant LODC-5 strain (P = 0.93). Our results showed that *A. castellanii* grew onefold less in the presence of *luxO* disruptant strain than the wild *V. mimicus* but no significant statistical difference was found (P = 0.99). Analysis of protease activity revealed that wild-type and mutant *V. mimicus* strains in the presence of amoeba did not have any significant differences in protease activity (P of t-test was 0.34) under the stationary phase of bacterial growth (data not shown).

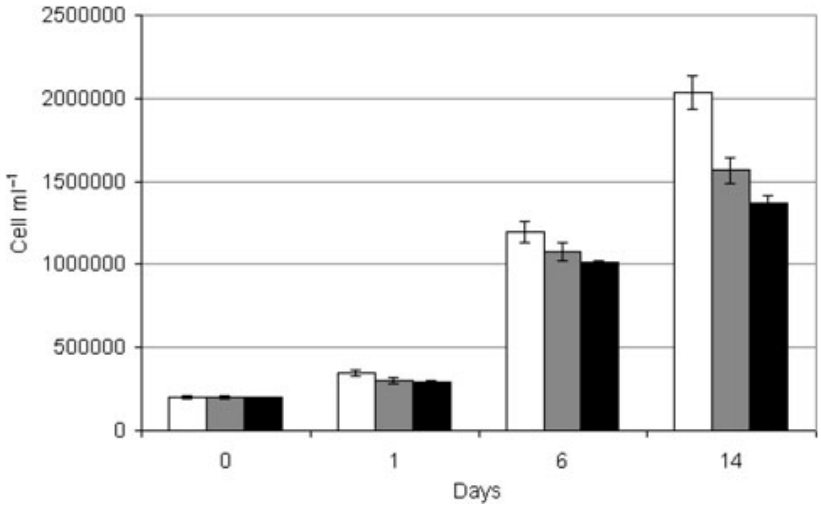

FIG. 10. Growth of *A. castellanii*. White staples indicate growth of *A. castellanii* in the absence of bacteria, grey in presence of wild type *V. mimicus* CS-5, and black in the presence of *luxO* disruptant mutant LODC5. Data indicate mean ± SD values of three repeated experiments.

4.2.2 Effect of *A. castellanii* on the growth of *V. mimicus* strains

To study growth of the bacteria alone and in co-culture viable counts of wild *V. mimicus* or its *luxO* mutant strain in the absence or the presence of *A. castellanii* was adjusted. We observed10-fold and 100- fold increase of wild *V. mimicus* and its mutant strain in the presence of *A. castellanii* after 1 day of incubation and bacteria survived for more than 2 weeks Fig. 11. Whereas in the absence of amoebae *V. mimicus* strains increased 10-fold during the first day followed by a decrease to 0.0 cfu ml-1 on day 6 Fig. 11. Student's *t*-test showed a statistically significant difference in the growth of wild and mutant *V. mimicus* in the presence or the absence of *A. castellanii* (*P*-values were 0.0001 and 0.00003). Interestingly, we observed the growth rate of the mutant strain has increased 10-fold more than the wild strain from day 1 to day 14 in Fig. 11, which may explain why *A. castellanii* grew onefold less in the presence of *luxO* disruptant strain than the wild *V. mimicus* Fig. 10.

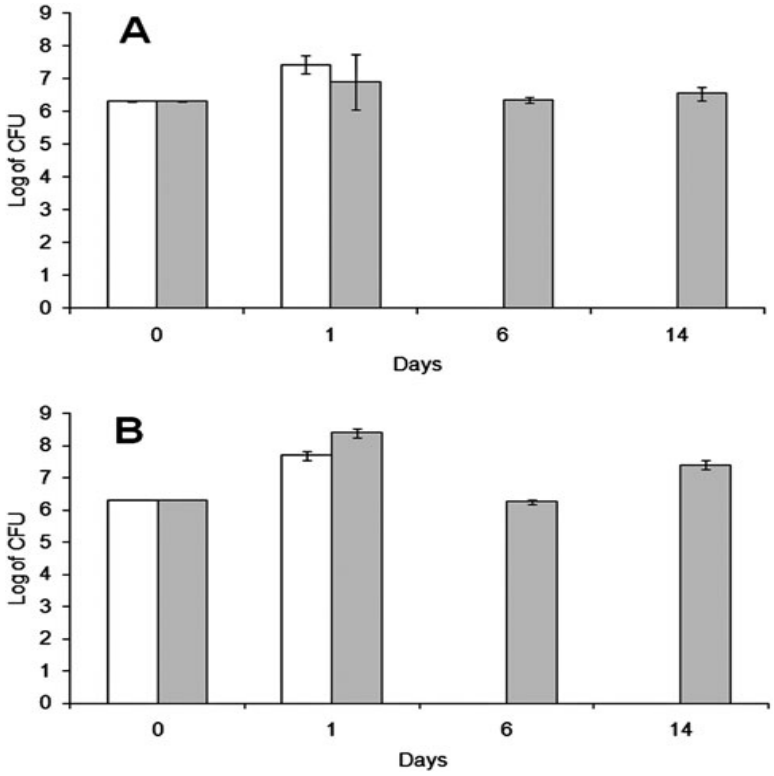

FIG. 11. Growth of *V. mimicus*. (A) Wild type *V. mimicus* CS-5 and (B) *luxO* disruptant mutant LODC 5. White staples indicate alone cultured bacteria and the grey co-cultured with *A. castellanii*. Data indicate mean values ± SD of three repeated experiments

4.2.3 **Intracellular growth, survival and localization of *V. mimicus***

Viable count of intracellular bacteria, show 3.0 x 10^2 ± 1.0 x 10^2 cfu ml-1 after 2 h of co-cultivation, 6.0 x 10^2 ± 2.0 x 10^2 cfu ml-1 after 4 h and to 1.0 x 10^5 ± 1.0 x 10^4 cfu ml-1 after 24 h. The bacteria survived intracellularly at 10^5 cfu ml-1 for more than 2 weeks.

As it was shown in Fig 12, transmission electron microscopy disclosed the localization of co-cultivated *V. mimicus* in the cytoplasm of *A. castellanii* cells compared with amoebae in the absence of *V. mimicus.* Amoebae in the presence of wild *V. mimicus* showed that bacterial cells were located in the cytoplasm of amoeba trophozoite after 1 day of co-cultivation Fig. 12B and after 3 days of co-cultivation Fig. 12D. The bacteria were also present in cysts after 1 day of co-cultivation Fig. 12C and after 3 days of co-cultivation Fig. 12E. Moreover, the bacterial cells were found in space between ecto- and mesocyst of the precyst stage after 3 days of co-cultivation Fig. 12F. The analysis also showed that the percentage of infected amoebae (trophozoite and cyst) was 57 ± 13 and that the number of bacteria inside amoeba cell was 37 ± 10.

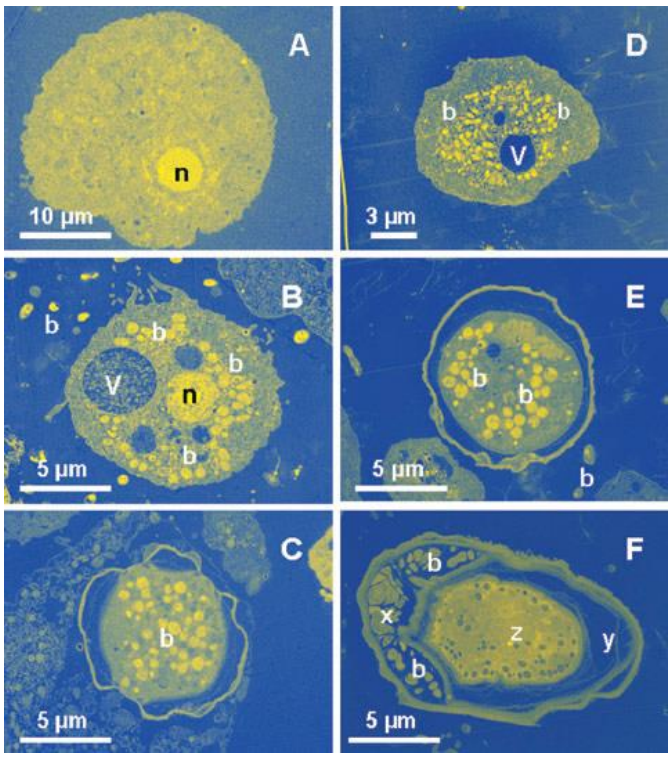

FIG. 12. Electron microscopy of the intracellular localization of *V. mimicus* CS-5 in *A. castellanii*. b, bacteria; n, nucleus; v, vacuole; x, ectocyst; y, mesocyct; z, endocyst. A. *Acanthamoeba castellanii* trophozoite in absence of bacteria. B. *Vibrio mimicus* CS5 localized in cytoplasm of *A. castellanii* trophozoite, 1 day after co-cultivation. C. *Acanthamoeba castellanii* cyst contains intracellular *V. mimicus* CS5, 1 day after co-cultivation. D. *Acanthamoeba castellanii* trophozoite contains intracellular *V. mimicus* CS5, 3 days after co-cultivation. E. *Acanthamoeba castellanii* cyst contains intracellular *V. mimicus* CS5, 3 days after co-cultivation. F. *Acanthamoeba castellanii* precyst contains intracellular *V. mimicus* CS5 in space between ecto- and mesocyst, 3 days after co-cultivation

4.2.4 Encystation of *A. castellanii* as a protective mechanism

Viable counts were performed for the amoebae and intracellular *V. mimicus*. The result of cell counts showed that the amoeba count was $1.6 \times 10^5 \pm 5.7 \times 10^3$ cfu/ml and that the all amoeba cells were cysts characterized by round cells with double walls having no nuclei at day 4. Whereas viable counts of intracellular *V. mimicus* after treatment with 1000 mg/ml gentamicin was $2.5 \times 10^4 \pm 7.0 \times 10^3$ cfu/ml on day 4.

4.3 TOXR OF *VIBRIO CHOLERAE* AFFECTS THE BIOFILM, RUGOSITY AND SURVIVAL WITH *ACANTHAMOEBA CASTELLANII*

4.3.1 Construction of a Δ *toxR* in *V. cholerae* and expression of outer membrane proteins.

An internal in-frame toxR deletion mutant in *V. cholerae* was constructed and examined for expression of outer membrane proteins by SDS-PAGE. The result showed that the *V. cholerae* toxR mutant strain expressed OmpT compared to the wild type strain, which expressed OmpU Fig 13.

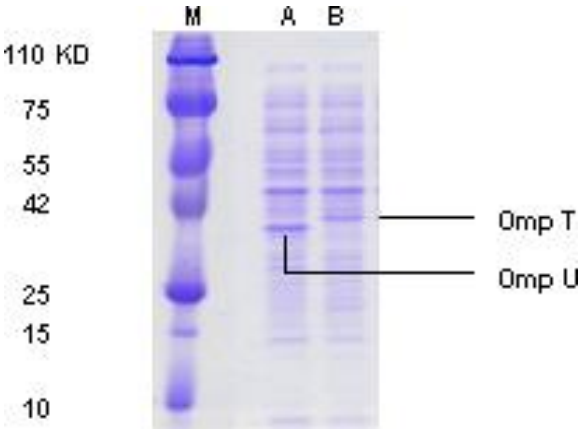

FIG. 13. Outer membrane proteins expression by SDS-PAGE gel from whole cell lysates. Lanes M, A and B represent marker, *V. cholerae* wild and toxR mutant strain. The gel confirmed that the wild V. cholerae expressed OmpU as it is usually activated by ToxR (A) while the constructed toxR mutant expressed OmpT (B).

4.3.2 Biofilm analysis and colony morphology of the *toxR* mutant compared with wild-type *V. cholerae*

Effect of ToxR in biofilm formation was studied at 30° C in LB broth. The photographical analysis showed that the wild type *V. cholerae* (Figure 14A upper panel) had repressed biofilm formation compared to the *toxR* mutant strain (Figure 14B upper panel). Moreover, it was found that the absorbance of the biofilm produced by the wild type *V. cholerae* (Figure 14A lower panel) and the *toxR* mutant strain (Figure 14B lower panel) were 1.1 ± 0.2 and 1.8 ±0.35, respectively. However, the spectrophotometry showed that the *toxR* mutant of *V. cholerae* had

enhanced biofilm formation, which was confirmed by the statistical analysis, since the difference in absorbance was statistically significant (p of t-test was < 0.05).

We also observed that the colonies of *V. cholerae* wild type strain were smooth (Fig 15 upper panel). Interstingly we found that 35 ± 10% of *toxR* mutant strain colonies was rugose (Fig 15 lower panel) and 65 ± 10% was smooth. The ability of each strain to form rugose colonies was significantly differed by x^2 test ($p < 0.001$).

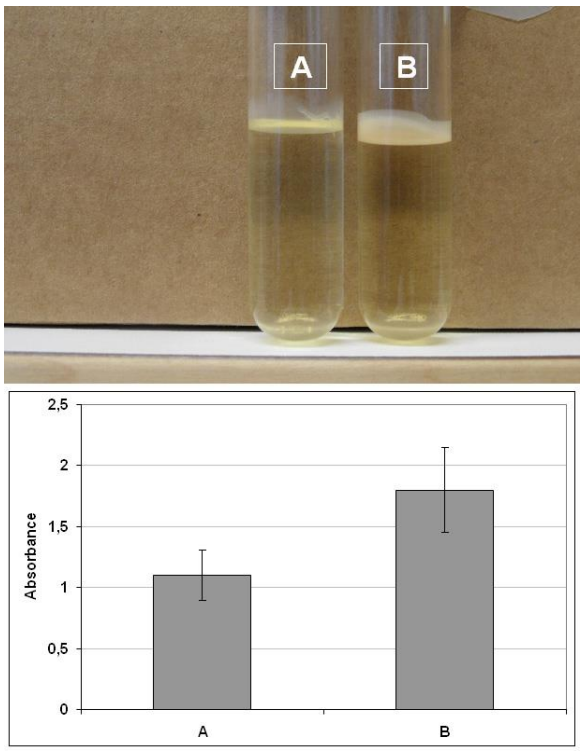

FIG. 14. Biofilm formation. Upper panel showed representative photographs of biofilms formed by wild type *V. cholerae* (A) and *toxR* mutant strain (B). Lower panel showed representative measurement of the absorbance, which is directly proportional to the concentration of biofilm associated crystal violet formed by wild type *V. cholerae* (A) and *toxR* mutant (B) strains. Data indicates mean ± SD of three independent experiments.

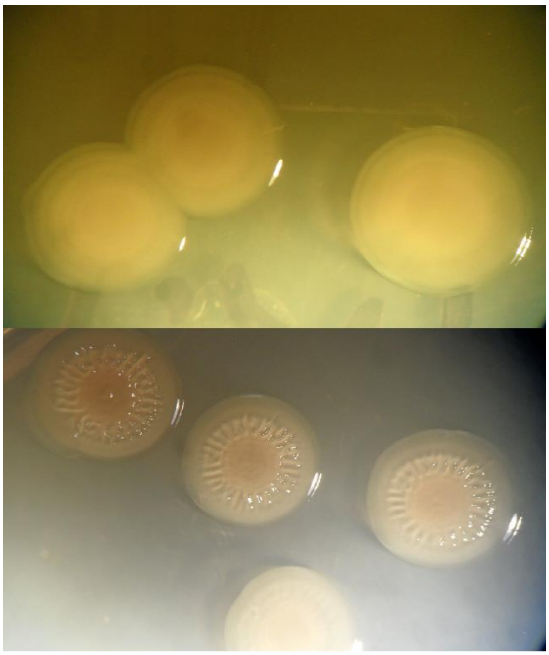

FIG. 15. Colony morphology of *V. cholerae* strains. Upper panel represents smooth colonies of wild type strain. Lower panel represents rugose colonies of *toxR* mutant strain.

4.3.3 Effect of ToxR protein on growth, survival and rugose colony formation of *V. cholerae* associated with *Acanthamoeba castellanii*

We found that the *V. cholerae* wild type and the *toxR* mutant survived differently at different temperatures. At 37°C the wild type strain grew to $1.3 \times 10^7 \pm 5.8 \times 10^6$ CFU/mL and survived 1 day in the absence of amoebae compared to $1.4 \times 10^2 \pm 5.5 \times 10$ CFU/mL and survived 3 days in the presence of the amoebae, whereas the *toxR* mutant strain died on the first day in the absence of amoebae but survived 1 day to $4.7 \times 10^5 \pm 4.0 \times 10^5$ CFU/mL in the presence of amoebae (Figure 16 upper panel). At 25° C the wild type strain grew to $7.5 \times 10^7 \pm 1.9 \times 10^7$ CFU/mL and survived 3 days, in the absence of amoebae compared to the *toxR* mutant strain, which decreased to $2.0 \times 10^4 \pm 1.0 \times 10^4$ CFU/mL and survived 2 days. Whereas in the presence of the amoebae, the wild type and the mutant strains grew to $3.3 \times 10^7 \pm 2.6 \times 10^7$ CFU/mL and $6.0 \times 10^7 \pm 3.0 \times 10^7$ CFU/mL and survived 10 days, respectively (Figure 16 lower panel). During the cultivation with the amoebae it was observed that the wild type and the *toxR* mutant strains did not form any rugose colonies at both 25° C and 37° C.

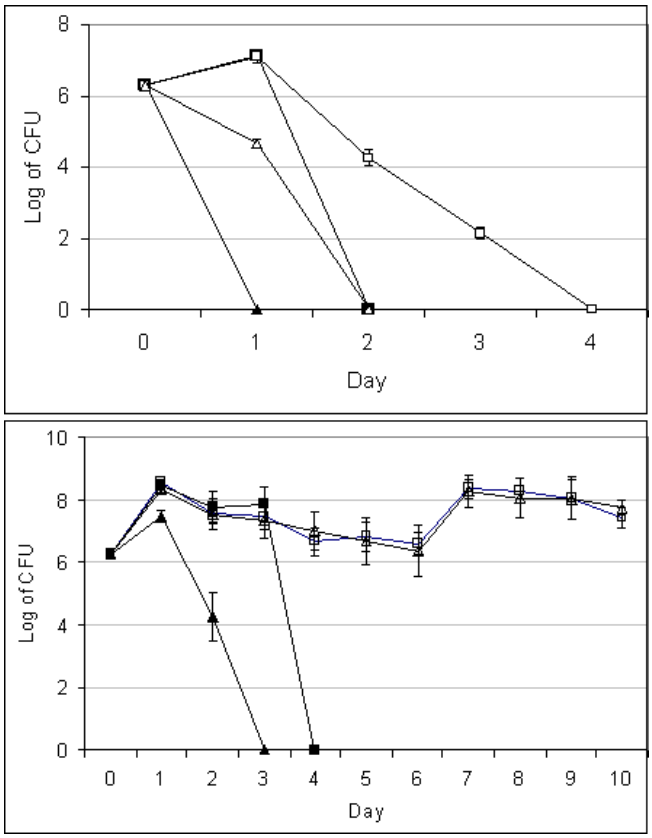

FIG. 16. Growth and survival of *V. cholerae* strains in absence and presence of *A. castellanii* at 37° C upper panel and at 25° C lower panel. Alone cultivated wild type *V. choleare* (■) and cultivated with A. castellanii (□). Alone cultivated toxR mutant strain (▲) and cultivated with *A. castellanii* (Δ). Data indicates mean ± SD of three independent experiments

4.4 OUTER MEMBRANE PROTEIN A SUPPRESSES SURVIVAL OF *VIBRIO CHOLERAE* AND OUTER MEMBRANE VESICLES INHIBIT VIABILITY OF *ACANTHAMOEBA CASTELLANII*

4.4.1 Adherence, uptake and intracellular growth

To estimate adherence of *V. cholerae* wild-type and *OmpA* mutant strain to amoeba cells, the percentage of each bacterial strain adhering to *A. castellanii* was determined and found to be 83.3% ± 2.1% and 73.3% ± 3.5%, respectively. The attachment to the wild-type and *OmpA* mutant *V. cholerae* was not statistically significant; *p* of *t*-test was 0.29.

To estimate growth and survival of the engulfed bacteria following gentamicin treatment and re-cultivation, the number of bacteria growing intracellularly was estimated by viable counts. Viable counts of uptaken wild-type and *OmpA* mutant of *V. cholerae* were $3.2 \times 10^3 \pm 1.6 \times 10^3$ and $4.0 \times 10^3 \pm 5.0 \times 10^2$, respectively Fig. 17.Viable counts of intracellular wild type and *OmpA* mutant of *V. cholerae* after 2 hours were $1.7 \times 10^3 \pm 1.2 \times 10^3$ and $1.4 \times 10^3 \pm 2.0 \times 10^2$,

respectively Fig. 17. Viable counts of intracellular wild-type and *OmpA* mutant of *V. cholerae* after 24 hours were 3.0 x 10^5 ± 1.0 x 10^5 and 1.5 x10^5 ± 5.0 x 10^4, respectively Fig. 17. The uptake and intracellular growth of the wild-type and OmpA mutant *V. cholerae* were not statistically significant; *p* of *t*-test was 0.68.

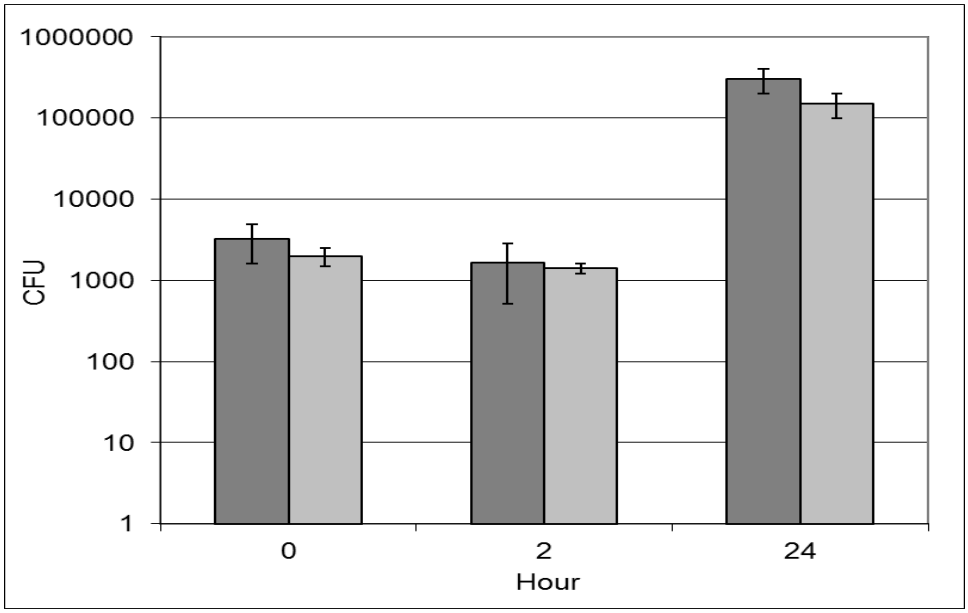

FIG. 17. Uptake, intracellular growth and survival of wild-type of *V. cholerae* (dark colored) and OmpA mutant strains (grey colored). Zero time is uptake of the bacteria by amoeba cells. Data represent mean ± SD from 3 different experiments.

4.4.2 Growth and survival of alone wild-type and *ompA* mutant of *V. cholerae*

Viable counts of alone cultivated wild type and *OmpA* mutant of *V. cholerae* in the absence of *A. castellanii* showed 1000000-fold increases after 1 day, respectively. Surprisingly the wild-type bacteria survived 3 days only when compared with the *OmpA* mutant *V. cholerae* which survived more than 2 weeks, and the viable count was 1,7 x10^3 ± 2,1 x10^2 in day 15 Fig. 18. The survival of the wild type and *OmpA* mutant of *V. cholerae* was statistically significant; *p* of *t*-test was 0.005.

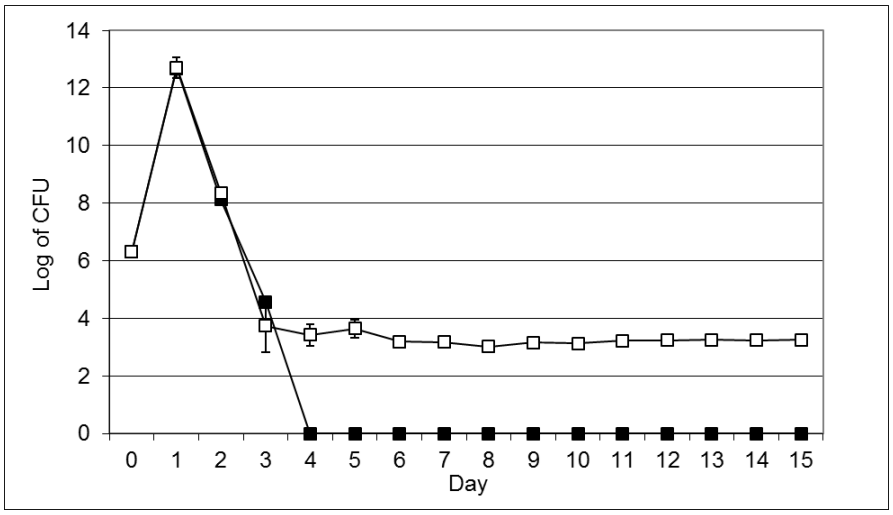

FIG. 18. Growth and survival of alone *V. cholerae* strains. The wild-type strain is filled squares and the *OmpA* mutant strain is empty squares. Data represent mean ± SD from 3 different experiments.

4.4.3 Growth and survival of co-cultivated wild-type and *OmpA* mutant *V. cholerae*

Viable counts of co-cultivated wild-type and *OmpA* mutant of *V. cholerae* with *A. castellanii* showed 1000-fold increases after 1 day, respectively. Surprisingly both wild- type and *OmpA* mutant of *V. cholerae* survived more than 2 weeks but their viable counts were different since the counts were $2.0 \times 10^5 \pm 1.0 \times 10^5$ and $4.1 \times 10^8 \pm 2.6 \times 10^8$ at day 15, respectively Fig. 19.

However, the presence of *A. castellanii* enhanced survival of both wild-type and *OmpA* mutant *V. cholerae*. The survival of the co-cultivated wild-type and *OmpA* mutant of *V. cholerae* was statistically significant; *p* of *t*-test was 0.0004.

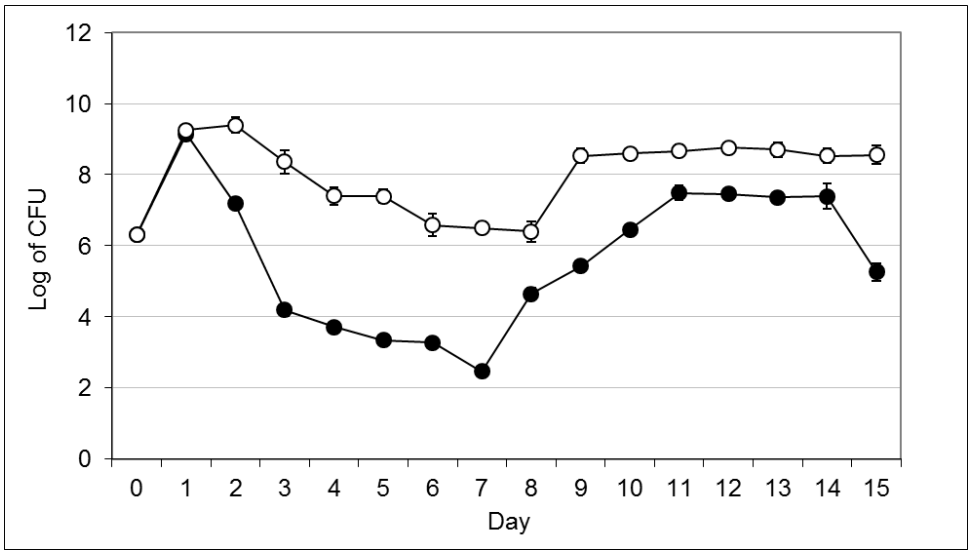

FIG. 19. Growth and survival of *V. cholerae* strains cultivated with *A. castellanii*. Co-cultivated wild-type *V. cholerae* is filled circles and *OmpA* mutant strain is empty circles. Data represent mean ± SD from 3 different experiments.

4.4.4 Growth and survival of co-cultivated *A. castellanii* with wild-type and *OmpA* mutant of *V. cholerae*

The number of viable *A. castellanii* co-cultivated with wild-type *V. cholerae* increased from 2.0 x 10^5 ± 0.0 cfu/ml to 9.3 x 10^5 ± 1.8 x 10^5 cfu/ml after 15 days compared to that of *A. castellanii* co-cultivated with *OmpA* mutant of *V. cholerae*, which decreased from 2.0 x 10^5 ± 0.0 cfu/ml to 1.3 x 10^4 ± 1.0 x 10^3 cfu/ml after 15 days Fig. 20.

The presence of wild-type *V. cholerae* enhanced growth of *A. castellanii* while presence of *OmpA* mutant of *V. cholerae* inhibited growth of *A. castellanii*; *p* of *t*-test was 0.04.

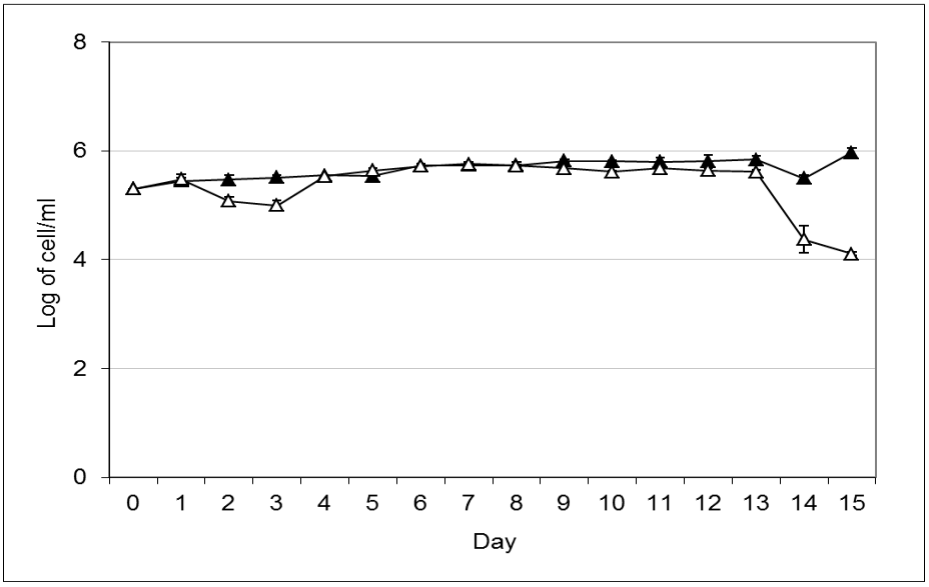

FIG. 20. Growth and survival of *A. castellanii* cultivated with *V. cholerae* strains. *A. castellanii* with wild-type of *V. cholerae* is filled triangles and *A. castellanii* with *OmpA* mutant strain is empty triangles. Data represent mean ± SD from 3 different experiments.

4.4.5 Production of outer membrane vesicles by *V. cholerae* strains and effect of the OMVs on viability of *A. castellanii*

Outer membrane vesicles were isolated from the wild-type and *OmpA* mutant strains as described in method section. The amount of vesicles released from the two strains was compared by measuring protein concentration. The protein concentration produced by the wild-type and *OmpA* mutant strains was 0.051 ± 0.024 mg/ml and 1.55 ± 0.051 mg/ml respectively Fig. 21.

The OMVs inhibited viability of the amoeba cells after 2 hours-incubation. It was found that viable count of *A. castellanii* incubated with OMVs from wild-type and *OmpA* mutant strain or with PBS were $8.7 \times 10^5 \pm 2.3 \times 10^5$, $8.1 \times 10^5 \pm 7.8 \times 10^4$ and $1.1 \times 10^6 \pm 1.3 \times 10^5$, respectively.

Viable count of the amoebae treated with OMVs from *OmpA* mutant of *V. cholerae* decreased significantly when compared to the treatment by PBS (*p* of *t*-test was 0.022) but that decrease was not significant when the amoebae were treated by OMVs from the wild-type *V. cholerae*, *p* of *t*-test was 0.052.

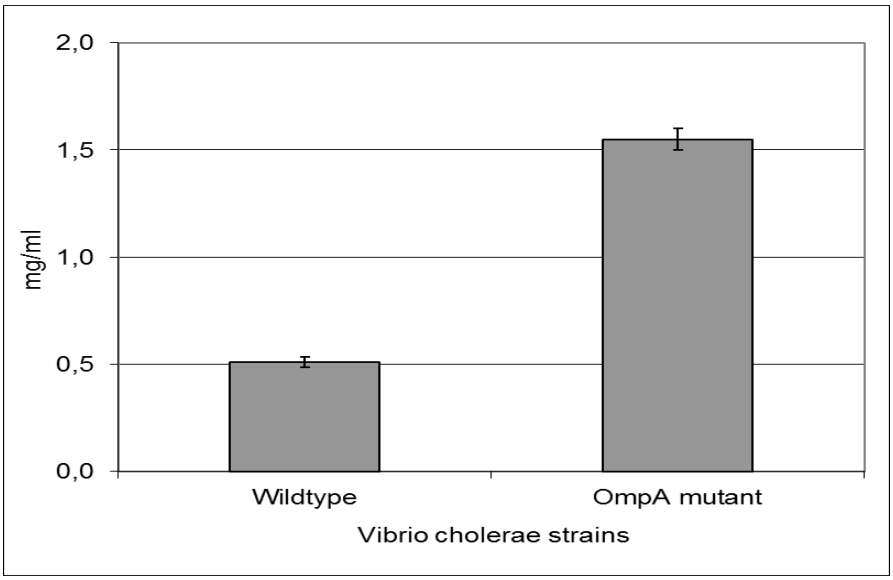

FIG. 21. Production of outer membrane vesicles by *V. cholerae* wild-type and *OmpA* mutant strain. Data represent mean ± SD from 3 different measurements.

5 DISCUSSION

Ability of bacteria to sense and respond effectively to environmental changes is crucial for their survival. Bacterial strategies studied at present would have been useful for survival or as defenses against overwhelming voracious predators in aquatic environments. The present thesis discusses the role of virulence factors on survival strategies of two different vibrio species named *V. cholerae* and *V. mimicus* in the presence of *Acanthamoeba castellanii*.

Melanin-like pigments are commonly produced by a variety of organisms including fungi, bacteria and helminthes (20). In some cases the ability of a microorganism to produce melanin has been related with pathogenicity and virulence for their hosts by reducing the pathogen susceptibility to host defense mechanisms (19, 20).

In paper I, by using transposon mutagenesis, we identified the gene locus of pigment over producing mutant on chromosome 1 in *V. cholerae* strain A1552; and the gene sequence was suggested to encode the enzyme oxidoreductase with the proposed designation HmgA .The *hmgA* gene of *V. cholerae* is located downstream of a putative 4-hydroxyphenylpyruvate dioxygenase gene and upstream of a conserved hypothetical protein, which is homologous to fumarylacetoacetate hydrolase family proteins from *Caulobacter crescentus* and *Mycobacterium tuberculosis* and to 2-hydroxyhepta-2, 4-diene-1, 7-dioate isomerase-related proteins in *S. solfataricus*, *Archaeoglobusfulgidus*, and *Thermoplasma acidophilum*. Trans-complementation test verifies that the pigment production was caused by the insertion mutation in the VC1345 open reading frame and the melanin pigment-producing mutant strain SNW29 was more resistant to UV irradiation than the WT *V. cholerae* strain.

Microarray expression profiling analysis of the *hmgA* gene and the neighboring genes such as VC1344 and VC1346 to VC1349, at the transcriptional level revealed that the expression of the VC1344-to-VC1349 gene cluster was influenced by the growth phase. Interestingly, two of the neighboring genes, VC1348 and VC1349, annotated to encode a putative response regulator and a sensor kinase, respectively, were observed to have the highest expression levels in the stationary phase, made us to speculate that this putative two-component system might be involved in sensing some environmental signal(s) and thus influences colonization ability. Further investigation by qRT-PCR analyses found that the mutation in either VC1348 or VC1349 caused reduced expression levels (to 50 to 70% of the WT level) of both VC1344 and VC1345. The finding that the expression level of the VC1344-to- VC1349 gene cluster was

increasing with increasing cell density prompted us to determine if the sigma factor RpoS might be required for expression of the tyrosine metabolic pathway and brown-pigment production in *V. cholerae*. We found that the transcription levels of the VC1344 and *hmgA* genes were reduced by 8-fold in the *rpoS* mutant of *V. cholerae* strain A1552 and slight increase in the levels of pigment production in the *hmgA rpoS* double mutant (SNW31) grown in the presence of tyrosine compared with the *hmgA* single mutant (SNW29). However, there were no differences observed in pigment production between the WT and the *rpoS* single mutant (SNW30) strain when grown either in the absence or in the presence of tyrosine.

In general *V. cholerae wild*-type 569B is non melanogenic in culture while its hypertoxic mutant strain, HTX-3, synthesized a pyomelanin, and suggested to be substantially more virulent than their nonmelanogenic parental strain (25, 74). However, the genetic alteration(s) of these hypertoxigenic or hypervirulent strains was not revealed. Interestingly with our *hmgA*::mini-Tn*5* mutant, we found elevated levels of secreted CTX and TcpA protein in comparison with the WT strain, A1552. This result clearly shows the increased level of TcpA protein and the main Tcp pilus subunit was the result of *hmgA* gene mutation, which explains the Putative link between the pigment formation and virulence factor expression. Two possible reasons could be explained for the elevated levels of TcpA in the *hmgA* mutant derivatives either the genetic expression of the *tcp* locus was increased or that the stability and turnover of the TcpA protein were altered but surprisingly, relative stability of TcpA in the mutant and parental strains did not reveal much difference in TcpA stability. The fact that the *hmgA* mutant also secreted a higher level of CTX would presumably favor the hypothesis that the mutant derivative is altered in its genetic expression of the virulence factors. A number of parameters, like temperature, pH, osmolarity, oxidative stress, amino acids, and bile, are known to modulate the expression of ToxR regulon, and such environmental signals exert their effects at different levels of regulatory cascade (30)**.** It was reported by Martin and Batkoff (75) that the HGA auto-oxidation at physiological pH can generate superoxide radicals and hydrogen peroxide (H_2O_2) in the metabolic disorder alkaptonuria in eukaryotic cells. Interestingly, we observed the elevated levels of TcpA with the increased concentration of H_2O_2. Colonization of the infant mouse intestine by the mutant strain also shows the fivefold increase than that by the WT strain. As colonization efficiency is also dependent on bacterial growth, the growth rates of strains A1552 and SNW29 in LB were determined, and we observed no growth rate difference between the WT and the mutant (data not shown). Taken together, this study reveals the melanin overproducing strain of *V. cholerae* could modulate the resistance of this bacterium to UV light and modest effect on virulence factor production thereby influence the colonization ability.

Recent studies have shown that *V. cholerae* has an enhanced growth in association with the free-living amoeba *A. castellanii* at 30° C (60, 61, 63, 64, 76) since *V. mimicus* shares similar properties with *V. cholerae* such as existence of virulence associated genes, namely cholera toxin and toxin co-regulated pilus genes (33) as well as LuxO protein that regulates protease activity (34)**.** We are interested to study the survival ability of *V. mimicus* along with *A. castellanii*.

In paper II the results found that *A. castellanii* grew one fold less in the presence of *luxO* disruptant strain than the wild *V. mimicus.* LuxO mutant possessed significantly higher protease activity than the wild strain during the log phase of bacterial growth, but no significant differences could be seen in the protease activity of the strains during stationary phase or after 24 h of growth (34)**.** In our study we found that the wild-type and mutant *V. mimicus* strains did not have any significant differences of protease activity in the presence of amoeba under the stationary phase of bacterial growth (data not shown). This may explain why the growth of amoebae in the presence of wild or mutant *V. mimicus* strains was not markedly affected. The growth of *V. mimicus* or its *luxO* mutant showed enhanced growth in the presence of *A. castellanii* and decreased to non-detectable levels in the absence of amoeba, which explains that the protease did not exert a deleterious effect on the growth of amoeba. It is noteworthy that the protease of *Pseudomonas aeruginosa* had no effect on the growth of *A. castellanii* cells, which were killed by type III-secreted proteins (77)**.** On the contrary, PrtV, an extracellular protease of *V. cholerae*, is necessary for killing of the worm *Caenorhabditis elegans* (78)**.**

Intracellular bacteria utilize different mechanisms to survive and multiply inside the host cells such as amoebae and macrophages. *V. cholerae* O1 and O139 survive in the cytoplasm of *A. castellanii* trophozoites, and bacteria can be found in cysts (63, 64, 79). *V. mimicus* cells were localized in the cytoplasm of amoeba trophozoites. The bacteria were present in cysts and the intracellular bacteria were viable for more than 2 weeks. Thus, *V. mimicus* showed an intracellular behaviour in the acanthamoeba–host model. Holden and colleagues found that 30–70% of *A. castellanii* contained intracellular *Legionella pneumophila* (80). In comparison, the present study shows that 40–70% of *A. castellanii* internalized *V. mimicus* and 25–55 bacteria could be counted per amoeba cell. It has been shown that *Acanthamoebae* benefit from the extracellular bacteria like *Escherichia coli* and *Klebsiella aerogenes* as a source of nutrients (81) , while the extracellular bacterium *P. aeruginosa* killed *A. castellanii* by the effect of type III-

secreted proteins (77) The reason for the difference in behaviour of different extracellular bacterial species is not known.

Encystation is a vital process of the amoebic life cycle. *Vibrio mimicus* grows inside *A. castellanii* and the amoebae protected the bacterium from antibiotic killing. The outcomes of this interaction strongly point out the intracellular behaviour of *V. mimicus* as well as the ability of *A. castellanii* to host the bacterium in aquatic environments. It has become increasingly apparent that bacterivorous predators such as free-living protozoa and nematodes could be exploited as model systems to gain significant insight into the pathogenesis of environmental bacteria (82). *Vibrio mimicus* is primarily an extracellular enteropathogen, and thus it will be interesting to evaluate the status of the transcriptome of this bacterium during intracellular growth in *A. castellanii*. In this regard, our co-cultivation model will serve as a valuable tool, which requires further investigation.

V. cholerae virulence factors such as, CT and TCP are coregulated by a transcriptional regulator ToxR, however, independently of the transcriptional activators TcpP and ToxT, modulates expression two outer membrane proteins OmpU and OmpT (83)**.** Transcription of *ompU* is induced by ToxR, whereas transcription of *ompT* is repressed by ToxR (39)**.** OmpU plays more protective role than OmpT against the bactericidal effects of bile salts and other anionic detergents (39) *V. cholerae* adopts several survival strategies in aquatic environments and can survive as free-living or in association with zooplankton and can build biofilm and rugose colonies (35, 37). A great variety of secreted or surface bound proteases and their specificities can contribute the bacterial-host interaction in different ways (84)

In paper III, we succeeded in constructing the internal in-frame *toxR* deletion mutant of *V. cholerae* strain, which express the ToxR repressed OmpT instead of the ToxR-activated OmpU. We disclosed its role in the environmental survival strategies of *V. cholerae* such as biofilm formation, switching from smooth to rugose colony morphotypes and association with *A. castellanii*.

Our results demonstrate that ToxR clearly affects biofilm and rugose formation since the differences in biofilm and rugose colony formation between *V. cholera* wild type and toxR mutant were significantly higher by t-test and χ^2 test, respectively. Viable count on blood agar reveals the effect of ToxR on growth and survival of *V. cholerae* associated with *A. castellanii*.

At 37º C *V. cholerae* wild type strain survived longer than the *toxR* mutant strain in the presence or absence of the amoebae. Interestingly, the association with *A. castellanii* enhanced survival of both bacterial strain but the wild type strain survived longer uncovering a role of ToxR in the survival of *V. cholerae* associated with protozoa in aquatic environment. Whereas in cultivation with the amoebae at 25° C, it was observed that both *V. cholerae* wild type and *toxR* mutant strains survived longer than 10 days. Despite *V. cholerae* wild type and *toxR* mutant strains survived more than 10 days in cultivation with the amoebae at 25° C, these strains did not form any rugose colony indicating that the bacteria avoided starvation. However, presence of the amoebae might enrich the cultivation medium and viable count of the bacteria was performed on enriched plates (blood agar plates).

Analogies to rugosity can be found in a number of other bacterial species, including the expression of alginate by mucoid strains of Pseudomonas aeruginosa and the expression of an adhesive EPS by members of the marine genus (38). Spontaneous and reversible variation in cell-associated and cell-free EPS production represents an optimal adaptive mechanism that facilitates survival in stressful environments (85). Although, over 20 genes are co-ordinately controlled by the ToxR regulon (86) the mechanism of switching is currently under study and it seemed to be regulated by exopolysaccharide related phase variation. However, role of ToxR was found to be critical for *V. cholerae* bile resistance, virulence factor expression, and intestinal colonization (39, 40). Surprisingly, ToxR homolog from *V. anguillarum* was found to regulate its own production, bile resistance, and biofilm formation (87) which might emphasis the regulatory role of ToxR in the expression of virulence factors for Vibrio species.

V. cholerae utilizes several survival strategies in aquatic environments such as biofilm formation, switching from smooth to rugose colony morphotypes and association with the free-living amoebae. Studies have shown that *V. cholerae* has an enhanced growth in association with *A. castellanii* (63, 64, 88) and both microorganisms are detected in same water samples from cholera endemic area(76). Paper IV investigated the role of OmpA protein and the OMVs released by *V. cholerae* on survival and interaction of the bacterium with eukaryotic host *A. castellanii*.

Here, the internal in-frame deletion of *OmpA V. cholerae* strain A1552 O1 El Tor Inaba succeeds to survive longer and produce significantly more amount of OMVs compared to the wild-type strain confirming previous result by Song and co-authors, who suggested that the lack of OmpA protein led to more production of OMVs (44). An interesting finding of current paper is that OmpA suppresses survival of wild-type *V. cholerae*, when cultivated alone. To

investigate effect of OMVs on the amoebae, *A. castellanii* cells were incubated with suspension of isolated OMVs in PBS for 2 hours, which resulted in a decreased viability of *A. castellanii*. This might suggests a virulence role of the OMVs towards the amoebae.

Interaction of *V. cholerae* strains with *A. castellanii* involved attachment of bacteria to the amoeba cells, engulfment, intracellular growth and survival inside the amoebae. Surprisingly, the differences between engulfment, intracellular growth and survival of the wild-type and *OmpA* mutant of *V. cholerae* were not statistically significant. In this context, Abd *et al* (61) reported that the capsule and LPS O-side chain did not affect engulfment, intracellular growth and survival of *V. cholerae* O139 when interacted with *A. castellanii* (61).

The interaction also showed that presence of *A. castellanii* enhanced survival of both wild-type and *OmpA* mutant of *V. cholerae* in agreement with the previous interaction of *A. castellanii* with wild-type *V. cholerae* O139, the capsule mutant strain and the capsule/LPS double mutant strain that enhanced survival (61). In spite of the fact that *V. cholerae* O1 El Tor possesses a mannose-sensitive haemagglutinin fimbria and *V. cholerae* O1 classical does not; they have enhanced survival and their intracellular growth in *A. castellanii* is not significantly different (63). All these facts may emphasise that the intracellular behavior of *V. cholerae* is a new survival strategy (61, 63).

On the other hand, presence of wild-type *V. cholerae* enhanced growth of *A. castellanii* while presence of *OmpA* mutant of *V. cholerae* inhibited growth of *A. castellanii* which might be due to overproduction of OMVs by the mutant strain. OMVs of *V. cholerae* have been suggested to promote the delivery of virulence factors to bacterial or eukaryotic cells (44). However, our result showed that OMVs inhibited viability of the amoebae which in turn suggest the possible role as virulence factors.

It was reported that OmpA levels inversely correlated with the amount of OMVs, and that the sRNA of *V. cholerae* were called as vibrio regulatory RNA of *OmpA* (VrrA) increased OMVs production comparable to loss of OmpA, since VrrA positively regulates the release of OMVs by down regulating of OmpA protein. (44)

OMVs formation has been suggested to be linked to turgor pressure of the cell envelope during bacterial growth (89). Gram-negative bacteria have developed many strategies to enable active virulence factors to gain access to the extracellular environment, typically the tissues or bloodstream of the host organism (90).

Vesicles are means by which bacteria interact with prokaryotic and eukaryotic cells in their environment. Biochemical analysis and functional characterization of pathogen-derived outer membrane vesicles reveal that this secretory pathway has been taken by pathogens for the transport of active virulence factors into host cells (54). The ability of OMVs to fuse with bacterial membranes and host cells to deliver content into the cytosol for directed intercellular transport of particular bacterial virulence factors into host cells and tissues (54, 91-93). Further investigations will shed more light on the function of the vesicles and their interaction with host cells.

In conclusion the *OmpA* mutant of *V. cholerae* expressed more OMVs and survived longer than the wild-type *V. cholerae*. The amount of OMVs isolated from *OmpA* mutant strain was high enough to inhibit viability of the amoebae suggesting a virulence role for OMVs. Surprisingly, co-cultivation with *A. castellanii* enhanced survival of both wild-type and *OmpA* mutant strains indicating that *V. cholerae* might be adapted to survive better in association with eukaryotes.

6 CONCLUDING REMARKS

In summary, the work presented on this thesis based on the role of virulence factors on environmental survival strategies of *V. cholerae* and *V. mimcus* has demonstrated that

1. Melanin pigment plays a role in virulence factor expression of the bacteria such increased resistance against UV- light and colonization ability.

2. *V. mimicus* shows an intracellular growth and survival in *A. castellanii*

3. The regulatory protein ToxR of *V. cholerae* suggests a new role in the expression of OmpT/OmpU and in the formation of biofilm, switching of morphotypes from smooth to rugose colony and in association with the free living amoeba *A. castellanii*.

4. The outer membrane protein A of *V. cholerae* suppressed the survival of alone cultivated wild-type *V. cholerae* and has no effect on attachment, engulfment and intracellular growth of *V. cholerae* with the amoebae, whereas *ompA* mutant released more outer membrane vesicles and inhibits the viability of amoebae.

7 ACKNOWLEDGEMENTS

First and foremost I thank to him who alone does great wonders!! Almighty God! You are my strength and my hope; I thank you for your glory and for giving me enough strength to accomplish my dreams.

My most sincere gratitude to **Prof. Gunnar Sandström**, for giving me the opportunity to work in his group. I am very lucky to have such a wonderful group leader. Your positive attitude always boosts me with new hopes and helps me to overcome all the frustrations of unsuccessful experiments and discussions. Thank you very much for the support and freedom you have given me at all levels of my PhD, which helps me to work on my own and also for providing me enough support to attend national and international conferences, which really helped me to think beyond my scientific knowledge and gave me new horizons to pursue my career in research. Thanks for having nice times during lunch hours. Apart from research, I have learnt a lot from you, which I am sure will, helps me at different stages of life.

My supervisor **Hadi Abd**, thank you for your immense supervision and trust on me. I appreciate your concern, guidance, motivation, encouragement and valuable support during all these past years without whom this thesis wouldn't be finished. Your practical skills, profound knowledge and continuous help from designing experiments to analyzing the results helped me becoming independent at the later stage of my research. Your inspirational thoughts, ideas and discussions led me to think innovatively, to enhance my knowledge and to improve my presentation skills. Apart from all this, your genuine attitude helps me to learn a lot not only in the research field in other aspects of life also. Thanks for being my supervisor.

I would like to extend my thanks to my co-supervisor **Amir Saeed,** for his helpful suggestions and discussions throughout my research. I had a great time with you during lunch hours and to share the same office where we had discussions about research and life. Thank you for your guidance and motivation to continue my path as a scientist.

My sincere thanks to **Prof. Andrej Weintraub,** Head of Doctoral studies, Department of Laboratory Medicine, for helping me with the documentation and necessary guidance. I appreciate your friendly attitude and for creating a healthy environment in the department.

Thank you very much for your valuable suggestions and guidance during the seminars. I am not happy to miss the Friday breakfast meetings.

I would like to Acknowledge **Prof. Carl Erik Nord, Matti Sallsberg** for their support in the department and valuable discussions during the seminars.

Prof. Amit pal, National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. Amit Ji, I have no words to express my gratitude; I would not be here today without your help.

I am grateful to **Monica Hammerberg** for her kind assistance during the starting days of my PhD at SMI with office work and accommodation.

Gudrun Rafi, for nice secretarial service, complicated paper work and shadow economy.

A special thanks to **Naradja**, for helping me with the most important thing….. "Accommodation" and making my stay easier to my work place.

I am extremely grateful to the **Swedish Civil Contingencies Agency (MSB)** for the scholarship that I have been granted during my years as a PhD student.

I am happy to acknowledge all the former and present members of SMI and Laboratory medicine. **Lek**, for sharing lunch and experiences about life. **Monica** and **Lisa** for their valuable assistance in the lab. My present and former colleagues, **Salah shanan-**you are very nice person**. Salah, Mohammed, Waleed** for the support and friendly attitude. Going to miss Arabic conversations. The young generation**- Haifa, Jhonel, Santo-** you are good colleagues. **Omar**, for warm greetings and wishes all the time. **Sonal, Subha, Vasu**- you are very nice Indian colleagues. **Jenny, Halime**- thanks for warm greetings, sweet smiles in the corridor. **Marita ward**, for helping me with the paper work**. Mamun, Samir, Lars, Fredrik, Gustaf, Jessica**, **Markus, Sepideh** for their corporation and making my stay pleasant at the department.

I am gratefrul to **Prof. Sun Nyunt Wai**, Department of Molecular Biology, Umeå University, a special person in my life for allowing me to work in her group. Her never ending ideas, enthusiasm, respect and dedication towards her work taught me a lot. I thank her for introducing me to Molecular Genetics. You inspired me a lot in the aspects of research.

Karolis, my first teacher working with *Vibrio cholerae*. Thank you for your valuable time and patience, I had a great time working with you. **Pramod-** Its nice working with you. **Tiyanyan**

Song- my elder sister, miss you……**Connie**, you are very nice person. **Anja-** you are so sweet…..**Barbro, Annika, Stina, Anna, Takahiko, Claudia, Berit, Hyun-sook, Jonas, Edmund** thank you for being nice to me during my stay in Umeå

I would like to take the opportunity to thank my Swedish family in Umeå. **Barbro** and **Steg Marklund** my swedish grandparents, thank you very much for your love and care during the beginning days of my life in Vännäs, Sweden. **Anders** and **Maria** for introducing me to the Church family. My Swedish parents **Henric** and **Gunilla Staxäng**- you are very nice persons. I will never forget your love and care. **Helena, KjellOla, Bengt Lunnergard, Jan Nilson, Jens and Sonia Lunnergard** for your love and concern as a family. **Tommy Björn,** a special person, words are not enough to express my gratitude. God bless your family.

My church family in Stockholm, pastor **John** for your warm welcome. Very special people, **Maria, Alex**- for your constant support and whole hearted solidarity. You are always close to my heart. **Sussan, Tsigereda, Ingrid, Ike -** thank you for your love. Cell-group - **Jörg, Angilika** - thank you very much for your support and prayers. **Arnold, Vivian, Alex, Minna, Julian**, **Winnie**- for having nice fellowship and prayers.

My words of thanks to the Indian friends -**Yasin** uncles family for organizing interactive programs on several occasions that makes me feel home . **Radhika** and family-thank you for your care, concern and the dinners we had to gather. Thanks for inviting us to summer house during every summer. Special thanks to **Ananth, Suhitha** for taking care of me during my pregnancy and whenever there is need. **Lalit, Sujatha**- thanks for your hospitality and useful advises for life. My love to **Archit**. Special thanks to **Sreenivas** and **Madhavi** for helping me with lunch during the most difficult times and for memorable get-togethers. Blessings to **Pranavi** and the little guy. **Tirupathi** and **Srujana**- young couple, thanks for your love and concern. I wish you both a very blessed future. **Venkat, Jane**- you are special!!!……thank you very much for your prayers and love. Blessings to **Amulya**. Special thanks to **Blesson, Blessie**- for your prayers and love. **Suby**- your love and care always remains to my heart. Best wishes to your family. My close friends and memorable persons forever….. **Priya, Chandi, Haritha**, **Swapna, Aasritha** for all the memorable days we spent together and for the encouragement and support. Miss you and love you all…

Unuru and Family- Words are not enough to express my gratitude. I thank God for you. My wishes and blessings are always there with you…..

My sincere thanks to all of my teachers in India who put their effort in letting me to stand here now as a PhD.

I am deeply indebted to my parents. Mom, Dad (**Prabhakar Rao, Ruth Mary**) – Thanks for giving me this life. I thank God for having such a wonderful parents-for their unselfish love, blessings and support throughout my life. My love to you both!!! I have no words and these pages are not enough to express my gratitude for the support, strength you have given me when I am away from you and for being with me from KG to PhD.

I also convey my gratitude to my parents in- laws (**Rev. Abraham Sudhakar, Mary Grace**) - for their constant blessings and prayers. Brother-in-laws (**David and John Billa**), Sister- in-laws (**Sudha, Madhu**) for your love and support during these years. My blessings to the little ones - **Nissi, Hassi** and **Jason**. Near and dear ones, those who are there behind the scenes have encouraged me and supported me at different steps of my life.

My love to my memorable Grandparents!!!

Last but not least, a very special person and the God gave sunshine of my life, my beloved husband **Sanjay**, without whom my venture would have been incomplete. Ammu, finally time has come……. Your love, affection, prayers and moral support made me to pass through all the hurdles I have faced during my PhD. Without you being in my life, I would have been incomplete and would have never stood anywhere. Thanks for the struggles and sacrifices you have done for us, for being at my side constantly showing me the true perceptions and how great the little things can be and for being my better half. Love you….

Finally, my sweet little daughter, **Seona,** my princess!! my joy!! who is two years old. She gave me so much affection and hundreds of reasons to smile that relieved all of my pain, tension's and worries. I hope Mommy did not bother you and Pappa when she is busy with her studies. I am sure you both will understand and I will wait for the day to come when you are big enough to read this.

Soni

8 REFERENCES

1. Euzeby JP. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int J Syst Bacteriol. 1997 Apr;47(2):590-2.

2. Popovic T, Olsvik. Ø, Blake PA, Wachsmuth K. Cholera in the Americas: Food borne aspects. J Food Protect. 1993;56:811-21.

3. Tauxe RV, Mintz ED, Quick RE. Epidemic cholera in the new world: translating field epidemiology into new prevention strategies. Emerg Infect Dis. 1995 Oct-Dec;1(4):141-6.

4. Wachsmuth I, Blake P, Olsvik O. Vibrio cholerae and cholera: Molecular to Global Perspectives. ASM Press, Washington, DC 1994.

5. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. Lancet. 2004 Jan 17;363(9404):223-33.

6. Kaper JB, Jr.Morris JG, Levine MM. Cholera. Clin Microbiol Rev. [Research Support, U.S. Gov't, P.H.S. Review]. 1995 Jan;8(1):48-86.

7. Attridge SR, Manning PA, Holmgren J, Jonson G. Relative significance of mannose-sensitive hemagglutinin and toxin-coregulated pili in colonization of infant mice by Vibrio cholerae El Tor. Infect Immun. [Comparative Study Research Support, Non-U.S. Gov't]. 1996 Aug;64(8):3369-73.

8. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-coregulated pili, and the toxR regulon are essential for Vibrio cholerae pathogenesis in humans. J Exp Med. [Research Support, U.S. Gov't, P.H.S.]. 1988 Oct 1;168(4):1487-92.

9. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ. Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci U S A. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 1987 May;84(9):2833-7.

10. Thelin KH, Taylor RK. Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by Vibrio cholerae O1 El Tor biotype and O139 strains. Infect Immun. [Comparative Study Research Support, U.S. Gov't, P.H.S.]. 1996 Jul;64(7):2853-6.

11. Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. Microbiol Mol Biol Rev. [Research Support, Non-U.S. Gov't Review]. 1998 Dec;62(4):1301-14.

12. Blake PA, Allegra DT, Snyder JD, Barrett TJ, McFarland L, Caraway CT, et al. Cholera--a possible endemic focus in the United States. N Engl J Med. [Case Reports]. 1980 Feb 7;302(6):305-9.

13. Weber JT, Mintz ED, Canizares R, Semiglia A, Gomez I, Sempertegui R, et al. Epidemic cholera in Ecuador: multidrug-resistance and transmission by water and seafood. Epidemiol Infect. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. 1994 Feb;112(1):1-11.

14. Colwell RR, Seidler RJ, Kaper J, Joseph SW, Garges S, Lockman H, et al. Occurrence of Vibrio cholerae serotype O1 in Maryland and Louisiana estuaries. Appl Environ Microbiol. [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. 1981 Feb;41(2):555-8.

15. Dobosch D, Gomez Zavaglia A, Kuljich A. [The role of food in cholera transmission]. Medicina (B Aires). 1995;55(1):28-32.

16. Mint E, Popovic T, Blake P. Transmission of Vibrio cholerae O1.Vibrio cholerae and cholera: Molecular to global perspectives. ASM press, Washington, DC. 1994.

17. Swerdlow DL, Mintz ED, Rodriguez M, Tejada E, Ocampo C, Espejo L, et al. Waterborne transmission of epidemic cholera in Trujillo, Peru: lessons for a continent at risk. Lancet. 1992 Jul 4;340(8810):28-33.

18. Zychlinsky A, Sansonetti P. Perspectives series: host/pathogen interactions. Apoptosis in bacterial pathogenesis. J Clin Invest. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. 1997 Aug 1;100(3):493-5.

19. Gomez BL, Nosanchuk JD. Melanin and fungi. Curr Opin Infect Dis. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. 2003 Apr;16(2):91-6.

20. Nosanchuk JD, Casadevall A. The contribution of melanin to microbial pathogenesis. Cell Microbiol. [Research Support, U.S. Gov't, P.H.S. Review]. 2003 Apr;5(4):203-23.

21. Weiner RM. Biopolymers from marine prokaryotes. Trends Biotechnol. 1997 Oct;15(10):390-4.

22. Wheeler MH, Bell AA. Melanins and their importance in pathogenic fungi. Curr Top Med Mycol. [Review]. 1988;2:338-87.

23. Carreira A, Ferreira LM, Loureiro V. Brown pigments produced by Yarrowia lipolytica result from extracellular accumulation of homogentisic acid. Appl Environ Microbiol. [Research Support, Non-U.S. Gov't]. 2001 Aug;67(8):3463-8.

24. La Du BN, Jr. Alcaptonuria and ochronotic arthritis. Mol Biol Med. [Review]. 1991 Feb;8(1):31-8.

25. Kotob SI, Coon SL, Quintero EJ, Weiner RM. Homogentisic acid is the primary precursor of melanin synthesis in Vibrio cholerae, a Hyphomonas strain, and Shewanella colwelliana. Appl Environ Microbiol. [Research Support, Non-U.S. Gov't]. 1995 Apr;61(4):1620-2.

26. Steinert M, Flugel M, Schuppler M, Helbig JH, Supriyono A, Proksch P, et al. The Lly protein is essential for p-hydroxyphenylpyruvate dioxygenase activity in Legionella pneumophila. FEMS Microbiol Lett. [Comparative Study Research Support, Non-U.S. Gov't]. 2001 Sep 11;203(1):41-7.

27. Coyne VE, al-Harthi L. Induction of melanin biosynthesis in Vibrio cholerae. Appl Environ Microbiol. [Research Support, Non-U.S. Gov't]. 1992 Sep;58(9):2861-5.

28. Chatfield CH, Cianciotto NP. The secreted pyomelanin pigment of Legionella pneumophila confers ferric reductase activity. Infect Immun. [Research Support, N.I.H., Extramural]. 2007 Aug;75(8):4062-70.

29. Denoya CD, Skinner DD, Morgenstern MR. A Streptomyces avermitilis gene encoding a 4-hydroxyphenylpyruvic acid dioxygenase-like protein that directs the production of homogentisic acid and an ochronotic pigment in Escherichia coli. J Bacteriol. [Comparative Study]. 1994 Sep;176(17):5312-9.

30. Nag S, Das S, Chaudhuri K. In vivo induced clpB1 gene of Vibrio cholerae is involved in different stress responses and affects in vivo cholera toxin production. Biochem Biophys Res Commun. [Research Support, Non-U.S. Gov't]. 2005 Jun 17;331(4):1365-73.

31. Nosanchuk JD, Casadevall A. Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. Antimicrob Agents Chemother. [Research Support, N.I.H., Extramural Review]. 2006 Nov;50(11):3519-28.

32. Ruzafa C, Sanchez-Amat A, Solano F. Characterization of the melanogenic system in Vibrio cholerae, ATCC 14035. Pigment Cell Res. 1995 Jun;8(3):147-52.

33. Shinoda S, Nakagawa T, Shi L, Bi K, Kanoh Y, Tomochika K, et al. Distribution of virulence-associated genes in Vibrio mimicus isolates from clinical and environmental origins. Microbiol Immunol. [Research Support, Non-U.S. Gov't]. 2004;48(7):547-51.

34. Sultan Z, Miyoshi S, Shinoda S. Presence of LuxS/AI-2 based quorum-sensing system in Vibrio mimicus : luxO controls protease activity. Microbiol Immunol. [Research Support, Non-U.S. Gov't]. 2006;50(5):407-17.

35. Wai SN, Mizunoe Y, Takade A, Kawabata SI, Yoshida SI. Vibrio cholerae O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. Appl Environ Microbiol. [Research Support, Non-U.S. Gov't]. 1998 Oct;64(10):3648-55.

36. Johnson LR. Microcolony and biofilm formation as a survival strategy for bacteria. J Theor Biol. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]. 2008 Mar 7;251(1):24-34.

37. Islam MS, Jahid MI, Rahman MM, Rahman MZ, Kabir MS, Sack DA, et al. Biofilm acts as a microenvironment for plankton-associated Vibrio cholerae in the aquatic environment of Bangladesh. Microbiol Immunol. 2007;51(4):369-79.

38. Wrangstadh M, Conway PL, Kjelleberg S. The production and release of an extracellular polysaccharide during starvation of a marine Pseudomonas sp. and the effect thereof on adhesion. Arch Microbiol. [Research Support, Non-U.S. Gov't]. 1986 Aug;145(3):220-7.

39. Provenzano D, Klose KE. Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes Vibrio cholerae bile resistance, virulence factor expression, and intestinal colonization. Proc Natl Acad Sci U S A. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 2000 Aug 29;97(18):10220-4.

40. Provenzano D, Schuhmacher DA, Barker JL, Klose KE. The virulence regulatory protein ToxR mediates enhanced bile resistance in Vibrio cholerae and other pathogenic Vibrio species. Infect Immun. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 2000 Mar;68(3):1491-7.

41. Kabir S, Mann P. Immunological properties of the cell envelope components of Vibrio cholerae. J Gen Microbiol. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 1980 Aug;119(2):517-25.

42. Kobayashi H, Uematsu K, Hirayama H, Horikoshi K. Novel toluene elimination system in a toluene-tolerant microorganism. J Bacteriol. [Comparative Study]. 2000 Nov;182(22):6451-5.

43. Delcour AH. Structure and function of pore-forming beta-barrels from bacteria. J Mol Microbiol Biotechnol. [Review]. 2002 Jan;4(1):1-10.

44. Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, et al. A new Vibrio cholerae sRNA modulates colonization and affects release of outer membrane vesicles. Mol Microbiol. [Research Support, Non-U.S. Gov't]. 2008 Oct;70(1):100-11.

45. Sugawara E, Nikaido H. Pore-forming activity of OmpA protein of Escherichia coli. J Biol Chem. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 1992 Feb 5;267(4):2507-11.

46. Torres AG, Kaper JB. Multiple elements controlling adherence of enterohemorrhagic Escherichia coli O157:H7 to HeLa cells. Infect Immun. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 2003 Sep;71(9):4985-95.

47. Kouokam JC, Wai SN, Fallman M, Dobrindt U, Hacker J, Uhlin BE. Active cytotoxic necrotizing factor 1 associated with outer membrane vesicles from uropathogenic Escherichia coli. Infect Immun. [Research Support, Non-U.S. Gov't]. 2006 Apr;74(4):2022-30.

48. Balsalobre C, Silvan JM, Berglund S, Mizunoe Y, Uhlin BE, Wai SN. Release of the type I secreted alpha-haemolysin via outer membrane vesicles from Escherichia coli. Mol Microbiol. [Research Support, Non-U.S. Gov't]. 2006 Jan;59(1):99-112.

49. Ellis TN, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol Mol Biol Rev. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review]. 2010 Mar;74(1):81-94.

50. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol. [Research Support, N.I.H., Extramural Review]. 2010;64:163-84.

51. Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. 2005 Nov 15;19(22):2645-55.

52. Loeb MR. Bacteriophage T4-mediated release of envelope components from Escherichia coli. J Virol. [Comparative Study]. 1974 Mar;13(3):631-41.

53. Yildiz FH, Schoolnik GK. Role of rpoS in stress survival and virulence of Vibrio cholerae. J Bacteriol. [Comparative Study]. 1998 Feb;180(4):773-84.

54. Rowbotham TJ. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. J Clin Pathol. 1980 Dec;33(12):1179-83.

55. Brown MR, Barker J. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. Trends Microbiol. [Research Support, Non-U.S. Gov't Review]. 1999 Jan;7(1):46-50.

56. Backer H. Water disinfection for international and wilderness travelers. Clin Infect Dis. 2002 Feb 1;34(3):355-64.

57. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin Microbiol Rev. [Review]. 2004 Apr;17(2):413-33.

58. Brown TJ, Cursons RT, Keys EA. Amoebae from antarctic soil and water. Appl Environ Microbiol. [Research Support, Non-U.S. Gov't]. 1982 Aug;44(2):491-3.

59. Martinez AJ, Visvesvara GS. Free-living, amphizoic and opportunistic amebas. Brain Pathol. [Research Support, Non-U.S. Gov't Review]. 1997 Jan;7(1):583-98.

60. Abd H, Valeru SP, Sami SM, Saeed A, Raychaudhuri S, Sandstrom G. Interaction between Vibrio mimicus and Acanthamoeba castellanii. Environ Microbiol Rep. 2010 Feb;2(1):166-71.

61. Abd H, Saeed A, Weintraub A, Sandstrom G. Vibrio cholerae O139 requires neither capsule nor LPS O side chain to grow inside Acanthamoeba castellanii. J Med Microbiol. 2009 Jan;58(Pt 1):125-31.

62. Sandstrom G, Saeed A, Abd H. Acanthamoeba polyphaga is a possible host for Vibrio cholerae in aquatic environments. Exp Parasitol. 2010 Sep;126(1):65-8.

63. Abd H, Saeed A, Weintraub A, Nair GB, Sandstrom G. Vibrio cholerae O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba Acanthamoeba castellanii. FEMS Microbiol Ecol. [Research Support, Non-U.S. Gov't]. 2007 Apr;60(1):33-9.

64. Abd H, Weintraub A, Sandstrom G. Intracellular survival and replication of Vibrio cholerae O139 in aquatic free-living amoebae. Environ Microbiol. [Research Support, Non-U.S. Gov't]. 2005 Jul;7(7):1003-8.

65. Yildiz FH, Schoolnik GK. Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci U S A. [Research Support, U.S. Gov't, P.H.S.]. 1999 Mar 30;96(7):4028-33.

66. Marmur J. A procedure for the isolation deoxyribonucleic acid from microorganisms. J Mol Biol. 1961;3:208–18.

67. Iwanaga M, Yamamoto K. New medium for the production of cholera toxin by Vibrio cholerae O1 biotype El Tor. J Clin Microbiol. [Comparative Study Research Support, Non-U.S. Gov't]. 1985 Sep;22(3):405-8.

68. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979 Sep;76(9):4350-4.

69. Kirn TJ, Lafferty MJ, Sandoe CM, Taylor RK. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by Vibrio cholerae. Mol Microbiol. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 2000 Feb;35(4):896-910.

70. Westermark M, Oscarsson J, Mizunoe Y, Urbonaviciene J, Uhlin BE. Silencing and activation of ClyA cytotoxin expression in Escherichia coli. J Bacteriol. [Research Support, Non-U.S. Gov't]. 2000 Nov;182(22):6347-57.

71. Hava DL, Camilli A. Isolation and characterization of a temperature-sensitive generalized transducing bacteriophage for Vibrio cholerae. J Microbiol Methods. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 2001 Sep;46(3):217-25.

72. Gardel CL, Mekalanos JJ. Alterations in Vibrio cholerae motility phenotypes correlate with changes in virulence factor expression. Infect Immun. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 1996 Jun;64(6):2246-55.

73. Wai SN, Lindmark B, Soderblom T, Takade A, Westermark M, Oscarsson J, et al. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell. [Research Support, Non-U.S. Gov't]. 2003 Oct 3;115(1):25-35.

74. Ivins BE, Holmes RK. Factors affecting phaeomelanin production by a melanin-producing (mel) mutant of Vibrio cholerae. Infect Immun. [Research Support, U.S. Gov't, Non-P.H.S.]. 1981 Dec;34(3):895-9.

75. Martin JP, Jr., Batkoff B. Homogentisic acid autoxidation and oxygen radical generation: implications for the etiology of alkaptonuric arthritis. Free Radic Biol Med. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 1987;3(4):241-50.

76. Abd H, Shanan S, Saeed A, Sandström G. Survival of Vibrio cholerae Inside Acanthamoeba and Detection of Both Microorganisms From Natural Water Samples May Point out the Amoeba as a Protozoal Host for V. cholerae. J Bacteriol Parasitol. 2011.

77. Hadi A, Bengt W, Amir S, Eva I, Kjell H, Gunnar S. Pseudomonas aeruginosa utilises its type III secretion system to kill the free-living amoeba Acanthamoeba castellanii. J Eukaryot Microbiol. 2008;55(3):235-43.

78. Vaitkevicius K, Lindmark B, Ou G, Song T, Toma C, Iwanaga M, et al. A Vibrio cholerae protease needed for killing of Caenorhabditis elegans has a role in protection from natural

predator grazing. Proc Natl Acad Sci U S A. [Research Support, Non-U.S. Gov't]. 2006 Jun 13;103(24):9280-5.

79. Amir S, Hadi A, Benjamin E, Gunnar S. Acanthamoeba castellanii an environmental host for Shigella dysenteriae and Shigella sonnei Arch Microbiol. 2009;191(1):83-8.

80. Holden EP, Winkler HH, Wood DO, Leinbach ED. Intracellular growth of Legionella pneumophila within Acanthamoeba castellanii Neff. Infect Immun. [Research Support, Non-U.S. Gov't]. 1984 Jul;45(1):18-24.

81. Weekers PH, Bodelier PL, Wijen JP, Vogels GD. Effects of Grazing by the Free-Living Soil Amoebae Acanthamoeba castellanii, Acanthamoeba polyphaga, and Hartmannella vermiformis on Various Bacteria. Appl Environ Microbiol. 1993 Jul;59(7):2317-9.

82. Hilbi H, Weber SS, Ragaz C, Nyfeler Y, Urwyler S. Environmental predators as models for bacterial pathogenesis. Environ Microbiol. [Research Support, Non-U.S. Gov't Review]. 2007 Mar;9(3):563-75.

83. Miller VL, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. J Bacteriol. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. 1988 Jun;170(6):2575-83.

84. Armstrong PB. Proteases and protease inhibitors: a balance of activities in host-pathogen interaction. Immunobiology. [Research Support, U.S. Gov't, Non-P.H.S. Review]. 2006;211(4):263-81.

85. Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type Escherichia coli: application to open reading frame characterization. J Bacteriol. [Research Support, U.S. Gov't, Non-P.H.S.]. 1997 Oct;179(20):6228-37.

86. Li CC, Merrell DS, Camilli A, Kaper JB. ToxR interferes with CRP-dependent transcriptional activation of ompT in Vibrio cholerae. Mol Microbiol. [Research Support, U.S. Gov't, P.H.S.]. 2002 Mar;43(6):1577-89.

87. Wang SY, Lauritz J, Jass J, Milton DL. A ToxR homolog from Vibrio anguillarum serotype O1 regulates its own production, bile resistance, and biofilm formation. J Bacteriol. [Comparative Study Research Support, Non-U.S. Gov't]. 2002 Mar;184(6):1630-9.

88. Sandstrom G, Saeed A, Abd H. Acanthamoeba-bacteria: a model to study host interaction with human pathogens. Curr Drug Targets. [Research Support, Non-U.S. Gov't Review]. 2011 Jun;12(7):936-41.

89. Zhou L, Srisatjaluk R, Justus DE, Doyle RJ. On the origin of membrane vesicles in gram-negative bacteria. FEMS Microbiol Lett. 1998 Jun 15;163(2):223-8.

90. Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D. Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev. [Research Support, Non-U.S. Gov't Review]. 2004 Dec;68(4):692-744.

91. Kondo K, Takade A, Amako K. Release of the outer membrane vesicles from Vibrio cholerae and Vibrio parahaemolyticus. Microbiol Immunol. 1993;37(2):149-52.

92. Loeb MR, Kilner J. Release of a special fraction of the outer membrane from both growing and phage T4-infected Escherichia coli B. Biochim Biophys Acta. [Comparative Study Research Support, U.S. Gov't, P.H.S.]. 1978 Dec 4;514(1):117-27.

93. McBroom AJ, Kuehn MJ. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Mol Microbiol. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. 2007 Jan;63(2):545-58.